CENTER for PRODUCE SAFETY

CPS 2012 RFP
FINAL PROJECT REPORT

Project Title
Die-off rates of human pathogens in manure amended soil under natural climatic conditions using novel sentinel chamber system

Project Period
January 1, 2013 – December 31, 2014

Principal Investigator
Keith Warriner
Department of Food Science
University of Guelph
519-824-4120 x 56072, kwarrine@uoguelph.ca

Co-Principal Investigator
Ann Huber
Soil Resource Group Inc
519-341-2176, ahuber@srgresearch.ca

Objectives
1) *Establish a steering committee to advise on trial methodology and disseminate results to stakeholder groups.*
2) *Verify the methodology of introducing and enumerating model pathogens into manure-amended manure.*
3) *Undertake field trials to determine die-off rates of model pathogens under variable climatic conditions.*
4) *Correlate pathogen die-off rates with soil type, season and climatic conditions.*

Funding for this project provided by the Center for Produce Safety through:
CPS Campaign for Research

FINAL REPORT

Abstract

The study determined the die off rates of Shiga Toxin producing *Escherichia coli* (STEC) and *Salmonella* in manure (dairy and swine) amended soil (loam and sandy loam). The inoculated manure and soil mix was placed into vials that were planted at depth (15 cm) or on the surface of field test plots. The trials were performed in the Spring (May) and Fall (October) with the temperature and soil moisture being monitored over the trial period. It was found that the die-off rates of STEC and *Salmonella* were variable so could not be fitted to linear or non-linear models. As a consequence the time for the population to decrease by 2 log cfu was recorded. By doing so it was found that the persistence of *Salmonella* was lower than that of STEC. Highest persistence of both pathogens was observed in loam soil at depth with lowest persistence on the surface of sandy soil. The rate of die-off of endogenous *E. coli* was lower than that of pathogens thereby suggesting it is a suitable metric for gauging the microbiological stats of soil. Although there was a relatively rapid die-off rates of STEC and *Salmonella* under field conditions persistent sub-populations were observed over the trial period (90-120 days). The levels of persistent strains was low and could only be detected by enrichment. Interestingly, the survival of STEC was greater in dairy manure amended soil compared to when swine manure was applied. The opposite was the case for *Salmonella*. The recorded die-off rates were more rapid in the Fall trial compared to Spring although further studies need to be undertaken to confirm the result. Under laboratory conditions the die off rates STEC and *Salmonella* were lower compared to those observed in the field. Here, high temperature and moisture increase the die-off rate of *Salmonella* although STEC were less stable under low moisture. Generic *E. coli* exhibited higher die off rates in laboratory microcosms compared to STEC or *Salmonella*. Further trials determined the fate of *Clostridium difficile* in field plots. It was found that endospores of 027 remained dormant whilst those of 078 (commonly encountered in the environment) decreased with time. Collectively, the results of the study have contributed to understanding factors which affect the die-off of pathogens in the natural environment. By considering the environmental and intrinsic factors it will be possible to develop predictive models that will be of use for growers with respect to manure management. A further important finding is that laboratory trials tended to overestimate the survival of STEC and Salmonella in manure amended soil. Consequently, undertaking die off studies in the environment may not be necessary in risk assessment.

Background

Major outbreaks of foodborne illness have been associated with fresh produce with any contamination acquired in the field being potentially be carried through to consumption (Warriner et al. 2009). Other crops grown on or in the ground such as melons and field cucumbers, carrots or onions are particularly vulnerable to pathogen contamination from soil borne sources. It is widely accepted that manure derived from animal production is the main source of human pathogens linked to fresh produce. Introduction can be indirect via irrigation water or direct by the application of manure onto fields for nutrient management. Although guidelines exist with respect to holding manure and then waiting a designated period prior to planting crops there is relatively little data to verify if the currently used guides are accurate with respect to pathogen die-off rates.

The die-off rates of pathogens (principally Shiga Toxin Producing *Escherichia coli*; STEC) has been reported by several research groups (Fremaux et al. 2008; Fremaux et al. 2010; Bolton et al. 2011; Ma et al. 2012; Erickson et al. 2014; Ibekwe et al. 2014). The general conclusions from the reported studies is that temperature, moisture, UV, soil type and endogenous microflora influence the persistence of pathogens in soil. Specifically, pathogen persistence is enhanced through high organic loading, low temperature and high moisture, in addition to protection against solar UV. With respect to resident microflora, there is tentative evidence that presence of *Actinobacter* and *Acidobacter* correlate with STEC survival although causation remains to be confirmed (Ibekwe et al. 2014). There is conflicting evidence on the strain dependency with reports of higher persistence of non-O157 STEC relative to the O157 serotype (Fremaux et al. 2010; Ibekwe et al. 2014).

Field studies have not always been consistent with respect to changes in pathogen populations in response to prescribed material application management practices. Difficulties in conducting these studies include temporal and spatial variability, and movement of microorganisms away from the initial placement of application with surface runoff or preferential flow. The sentinel methodology proposed for this study addresses these problems.

Understanding the effects of manure management and tillage practices on the survival of manure-derived pathogens is important in formulating recommendations for surface and ground water source protection and food safety. High levels of indicator organisms and pathogens in surface waters have been associated with agricultural operations, as has contamination of field grown fruits and vegetables when manure or NASMs have been applied to horticultural land.

Organic production systems standards require that “manure application shall be designed to ensure that manure application a. does not contribute to the contamination of crops by pathogenic bacteria, b. minimizes the potential for run-off into ponds, rivers and streams, c. does not significantly contribute to ground and surface water contamination. The non-composted solid or liquid manure shall be a. incorporated into the soil at least 90 days before the harvesting of crops for human consumption that do not come into contact with soil; b. incorporated into the soil at least 120 days before the harvesting of crops having an edible part that is directly in contact with the surface of the soil or with soil particles.”

The effects of manure application practices on nutrient losses have been extensively studied and it is generally concluded that incorporation reduces losses, particularly of nitrogen. OMAFRA manure application recommendations have been formulated on this basis. The number of studies on the effects of these practices on pathogen survival under different conditions (warm summer, sub-zero winter, and cool wet spring and fall) is limited, but most suggest that incorporation reduces pathogen die-off rates. This would indicate a contradictory effect of reduced nutrient losses but longer residual times for pathogens. For example, Hutchison (Hutchison et al. 2005) et al. applied manure spiked with *Salmonella, Listeria, Campylobacter*, and *E. coli* O157, and found that incorporation resulted in slower die-off of the pathogens likely because of reduced exposure to UV, heat, and drying effects. Surface application or delayed incorporation reduced

the decimal reduction time by nearly half. Hutchison (Hutchison et al. 2005)) did not find any significant difference in the pathogen die-off rate during winter relative to the spring, but that was not unexpected given the consistent soil temperature during both seasons. Nevertheless, there was significant variation in the pathogen loading of soils, which may have been attributed to the heterogeneous distribution of the target bacteria resulting from rainfall-facilitated movement.

Studies have not always been consistent with respect to the magnitude or even direction of change in pathogen populations in response to incorporation. Survival rates of *E. coli, Salmonella* and *Clostridium perfringens* spores were similar in surface-applied and incorporated biosolids (Holley et al. 2006; Eamens et al. 2011). Avery (Avery et al. 2004) compared surface application and injection, and concluded that injection reduced the survival time of *E. coli* O157. Field plot work in Ontario by Scott (Scott et al. 2006) indicated longer survival of *E.coli* with incorporation, and strain differences in survival and re-growth dynamics.

Several difficulties are encountered when examining the effect of management practices on the survival of pathogens under field conditions. There is large temporal and spatial variability in the size of the microbial populations within a field location, even following manure application. This variability will be a function of soil composition, macro- and micro pore structure, organic material, and variability of manure application. Furthermore, it is not expected that pathogenic organisms such as *Salmonella* and *Listeria* will be consistently present at detectable levels in field soils. Population changes in indicator organisms or pathogens can occur as a result of die-off, physical losses from surface runoff or movement down the soil profile, or addition of organisms from external sources (e.g. wildlife). Regrowth has also been shown to occur (Topp et al. 2009). This results in a complex system with high variability, making data from field studies difficult to interpret. Laboratory studies have often been used to impose more control on the test system, but information on survival derived from laboratory studies using highly consistent conditions of temperature and moisture etc. does not necessarily translate well to field conditions, where physical conditions fluctuate widely.

Sentinel chambers were also used by Robertson (Robertson and Gjerde 2006) to examine the effects of the Norwegian winter environment on the survival of *Cryptosporidium* oocysts and *Giardia* cyst. The authors concluded that shear forces generated during the repeated freeze thaw cycles disintegrated the cysts, which did not survive the winter.

A previous study by Huber et al. (2009, New Directions Project # SR9182) demonstrated the effectiveness of sentinel chambers in measuring indicator and pathogen decline rates in on-farm liquid and solid manure storages throughout two spring/summer and two fall/winter storage periods. The method enabled a) monitoring of a defined population throughout the storage period, and b) addition of specific pathogens to contained subsamples of manure held within the manure storages. Samples were held in containers that did not allow passage of microorganisms in or out of the sample, but did allow for the same changes in nutrient, moisture, oxygen, and pH

conditions that occurred in the surrounding material. Survival dynamics of the organisms examined (*E.coli, E.coli* O157, *Salmonella)* were shown to be comparable in the sentinel chambers and in the bulk manure, when manure was held under static conditions (i.e. no new manure added). A current study continues this research on manure storage management, focusing on liquid swine manure. Sentinel chambers have also been used by other researchers to examine changes in the dynamics of antibiotic resistance patterns in *E.coli* in liquid manure storages (Duriez and Topp 2007).

Research Methods and Results

Objectives

1) Establish a steering committee to advise on trial methodology and disseminate results to stakeholder groups.
2) Verify the methodology of introducing and enumerating model pathogens into manure-amended soil.
3) Undertake field trials to determine die-off rates of model pathogens under variable climatic conditions.
4) Correlate pathogen die-off rates with soil type, season and climatic conditions.

Methods

Steering Committee

- A steering committee was established with representation from OMAFRA, MOE, University of Guelph, industry groups and Dr T Suslow (UCDavis). The steering committee discussed the experimental approach along with the applied methodology.

Validation and verification of enumeration/detection methods

The pathogens used in the study were Shiga Toxin producing Escherichia coli (STEC), *Salmonella*, *Listeria monocytogenes* and *Clostridium difficile* endospores (Table 1).

The inoculation (pathogen-spiked manure mixed with soil) and recovery methods proved effective and repeatable. Three types of pathogens were selected as model pathogens to introduce in to the sentinel chambers: *E.coli*, *Salmonella*, and *Listeria*. Four-strains cocktails are being used for each pathogen type. Also, a cold pre-stress step was followed after incubation of the cultures to ensure high activity of the inocula. Several strains used in a previous study are being used in the current study to maintain consistency. Considering the increasing risk of non O157 *E.coli* infections two *E.coli* O26 and *E.coli* O145 have been added.

Field Trials

Perth loam and Hillsburg fine sandy loam were derived from fields the day before the trial date along with fresh manure from swine and dairy operations. The manure was mixed with the different soils at a ratio of 20g/kg soil along with the appropriate cocktail of pathogens. The inoculated manure soil mix was well mixed then dispensed into the sentinel vials.

The test plots were prepared by digging a 15 cm depth trench within the test plot and placing the vial containing the inoculated soil:manure mix (Figure 1A). For *C difficile* only one depth (15cm) was applied.

The vials were flagged and then the trench filled in with the appropriate soil as contained in the plot and vials. A further set of vials was placed on the subsurface and overlayed with a thin (2 cm) soil layer. Flags were placed on the plot to differentiate between the vials (Figure 1B). A weather station was established to collect continuous precipitation, soil moisture, and air and soil temperature data through the trial period (Figure 1C).

At each sampling point three sets of vials for each set were recovered and returned to the laboratory for microbiological analysis. At the sample time, moisture measurements and generic *E coli* levels were determined on non-inoculated controls within vials and from samples taken from the plot area. The trial was initiated on May (or October for the Fall trial) with samples being collected after 10 days then approximately monthly.

Laboratory Soil Microcosms

Laboratory trials were undertaken to determine the influence of temperature and moisture content on pathogen die-off rates. Moisture levels of the lab trial are set at 7%, 15% and 35% based on the range of measured soil moistures encountered in the field trials. Temperatures evaluated were 4°C, 10°C, 20°C, and 30°C.

The soil microcosms were prepared in 3 liter capacity plastic containers with a layer of gravel at the base for drainage overlaid with inoculated manure amended soils. The moisture content was adjusted to the appropriate level the core samples (1 cm diameter) withdrawn at t = o then periodically throughout the 60 day trial period. The moisture content of the samples were determined along with microbial levels.

Results

Steering committee
A steering committee was established that included the collaborating partners in addition to staff from the Ontario Cattlemen's Association, Ministries of Food & Agriculture (OMAF) and Environment (MOE), in addition to Dr Suslow from UCDavis. The main discussion points was to verify the experimental approach as outlined in the original proposal. It was also decided to include non-O157 Shiga Toxin Escherichia coli and Clostridium difficile given their increasing food safety significance.

Verification of microbiological techniques

Verification trials were undertaken by inoculating manure (liquid dairy and swine) amended loam or sandy loam soils. The pathogen cocktails were inoculated a levels within the range of 1 – 5 log cfu/g then recovered using the methods described in Table 1. The selective enumeration of both Salmonella and STEC could be achieved when initial levels were >2 log cfu/g. However, at lower levels the background microflora resulted in interference making identification of typical colonies problematic. To overcome the influence of background microflora the STEC and Salmonella strains were trained on nalidixic acid. Although the background microflora was not completely eliminated by the inclusion of nalidixic acid in the selective media it was sufficient to enable selective enumeration at levels of 1 log cfu/g.

Selective enumeration of *Listeria monocytogenes* proved problematic due to high background microflora even at relatively high levels (>4 log cfu/g). Attempts were made to transform with pRSET-BFP plasmid that encoded for GFP (Green Fluorescent Protein) and ampicillin resistance. However, in the absence of antibiotic selective pressure (i.e. when amended into manure amended soil) the plasmid label was readily lost. Molecular based techniques based on RT-PCR and isothermal PCR could detect *L. monocytogenes* in enrichment cultures but not quantitatively. Given that the primary objective of the project was to determine die-off rates of pathogens in manure amended soil it was decided to omit Listeria from subsequent field trials.

Pathogen die-off in manure amended soil

In the study of the pathogen die-off, five study variables are set as: pathogen type, soil type, manure type, seasonal manure application timing, and depth in soil. Two pathogens were selected as model pathogens to introduce in to the sentinel chambers: STEC and *Salmonella.* The die-off curves for the trials are presented in the annex A of the report.

In general, the die-off curves of *Salmonella* and STEC in the soil plots were characterized by non-linear kinetics with three or more phases. The initial phase was an initial rapid decline in pathogen levels with an occasional re-growth of *Salmonella* but not STEC. The die-off rate then progressively decreased the plateaued with a sub-resistant sub-population persisting through recovery of low levels of residual survivors over the 6 month trial period. The consequence of the multi-phasic die-off rates is that the classic first-order kinetic model (as used by others) could not be applied nor the standard non-linear models given the apparent heterogenous sub-populations present. Nevertheless, models are being developed using additional data derived from laboratory studies and those from previous trials. For the current report, it was deemed appropriate to define the time for the die-off to attain 2 or 3 log cfu reduction on the basis that low levels of residual pathogen would not likely represent a significant food safety issue although such an assumption requires to be verified.

The time to reach a 2 log cfu/g die off of the different bacterial types was found to be dependent on the soil type (sandy vs sandy loam), location (surface vs depth) and manure type (swine vs dairy). The general conclusions that can be drawn from the results is that the die off rates of pathogens introduced at depth into sandy loam soils exhibited the highest persistence compared to those located on the surface or in sandy soil. The only exception was the survival of STEC in swine manure that exhibited comparable survival regardless of location or soil type (Figure 2; Table 3).

Considering sandy loam at depth represents the worse-case scenario, it was observed over the trials that generic (non-pathogenic *E. coli*) exhibited higher persistence compared to both STEC and Salmonella. The results would suggest that generic *E. coli* would provide a suitable surrogate to monitor pathogen die-off. However, it should be noted that in the current study the *E. coli* was endogenous to the manure and hence better adapted to survival within the soil environment. A further interesting observation is that the STEC exhibited higher persistence in dairy manure compared to swine. In a similar manner, Salmonella appearing to be more adapted to soil amended with swine manure compared to that derived from dairies. It is tempting to speculate that the adaption of STEC to ruminants and Salmonella to pigs may also influence survival within the environment.

Survival of STEC and *Salmonella* on the surface of loam soil or within sandy soil. The same observation was also made for generic *E. coli.* It is likely that the low organic content along with the desiccation caused by the sand matrix negatively influenced the survival of bacterial cells along with the expose to solar UV on those vials on the surface.

Attempts were made to correlate the observed die-off rates with temperature (soil and environment) and soil moisture content of the field plots. However, the data could not be fitted to linear or non-linear kinetics due to the confounding factors. Therefore, to assess the influence of temperature and moisture a series of laboratory trials were undertaken.

Pathogen die-off rates in soil microcosms
Manure amended soil microcosms were prepared and inoculated with a STEC or *Salmonella* cocktail. Controls were run in parallel to determine the die-off rates of endogenous *E. coli* populations.

In general, rate of pathogen die-off under constant temperature and moisture conditions were constant (predictable), compared to field trials. In addition, the die off rates were longer than observed in the field which again would suggest that variation in environmental conditions

negatively affected pathogen survival. By taking the linear part of the die-off the rate of decline was calculated under constant temperature and moisture.

Both moisture and temperature affected the rate of pathogen or E. coli die off (Table 4). For Salmonella the highest rate of pathogen die off was under high moisture and temperature. Conversely, low moisture and temperature enhanced Salmonella persistence. The die-off kinetics of STEC followed a similar pattern in that the rate of inactivation increased with temperature. However, in general, the rate of inactivation was less dependent on moisture content apart from at the highest level applied (25%). Here, low moisture at high temperature (30°C) increased the rate of STEC inactivation. Generic *E. coli* followed the same pattern as STEC although importantly the rate of inactivation under different conditions was higher compared to that of the pathogen. This would raise a question on the suitability of generic *E coli* as a surrogate for pathogens given the higher die-off rates compared to STEC or *Salmonella.*

The results from the laboratory trials illustrated that the die-off of pathogens is dependent on the prevailing extrinsic factors. Although the data generated from laboratory trials could be modelled using first-order kinetics there are questions relating to how such die-off rate constants relate to those of pathogens in the natural environment. In this regard it is noteworthy that persistence of pathogens in laboratory trials was significantly higher than observed in the field. The difference is likely due to the fluctuations in temperature and moisture that combine to decrease pathogen viability.

Current efforts are being directed at building a predictive model based on the field and laboratory data.

Persistence of Clostridium difficile endospores in manure amended soil

Trials were performed to determine the persistence of *Clostridium difficile* endospores in manure emended soil. For this, the two most relevant ribotypes were inoculated into sentinel vials as previously described the placed at 15cm depth in both soil types. Ribotype 027 is commonly implicated in hospital acquired infections and continues to be the cause of epidemics within clinical settings. Ribotype 078 is rarely found in clinical settings although is highly prevalent in animal production facilities (especially pigs). The ribotype is also associated with community acquired infections which does not follow the same risk factors (being administered antibiotics and contact with clinical settings) as ribotype 027.

The vials were placed in test plots in May and sampled through to November. From the results it was found that endospores of 027 remained stable throughout the trial period. However, spores of ribotype 078 progressively decreased in loam soil and also in sandy loam after an initial lag period (Figure 4). Although not investigated further, it is likely that the organic matter in loam along with moisture and temperature resulted in spore germination with subsequent loss of viability of the subsequent cells.

The finding that ribotype 027 spores remained persistent maybe unexpected given that strains are not commonly recovered outside clinical settings. Yet, it is plausible that the success of 078 may be the ability of the strains to germinate under environmental conditions which then grow under the appropriate conditions.

Outcomes and Accomplishments

The primary objective of the study was to model pathogen die-off rates under natural conditions environment. In the current study the influence season (temperature and moisture) and manure type was taken into consideration, in addition to soil types (loam and sandy loam). From the results obtained the following conclusions can be made.

Standard culture based methods suffer from high background interference necessitating antibiotic resistant markers to be introduced into the test pathogenic strains. There are no current quantitative methods for enumerating *Listeria monocytogenes* in manure amended soil samples.

The die-off rates of STEC and *Salmonella* within environmental test plots follow multi-phase kinetics with a rapid initial decrease followed by re-growth on occasions then extended persistence beyond 90 days and in some instances 120 days.

The high variability in the data made it problematic in terms of determining inactivation rates using either linear or non-linear models. The presence of persistent sub-populations would also make constructing valid models problematic. Therefore, in the absence of specific inactivation data the time taken for pathogen populations to reduce by 2 or 3 log cfu. By taking this approach it was found that the persistence of both STEC and Salmonella was enhanced in loam soil at depth with rapid inactivation being observed on surface of sandy soil. It was also noted that STEC appeared to persist longer in soil amended with dairy manure compared to swine manure.

It appeared that pathogen die-off was more rapid in the Fall compared to the Spring although further trials are required to confirmed this conclusion.

Laboratory trials confirmed the influence of moisture and temperature on the inactivation kinetics of STEC and *Salmonella* in manure amended soil. Under conditions of constant temperature or moisture the inactivation kinetics followed diphasic behavior but could be modelled using first order kinetics. Persistence was influenced by both moisture and temperature. For *Salmonella* the highest inactivation rates were observed at high moisture (25%) and temperature (30°C). In the case of STEC the highest die-off rates were recorded at high temperature but low moisture. Pathogen die-off rates were lower compared to those observed in the environment.

In practice, the microbiological status of soil is determined by screening for generic (non-pathogenic *E. coli*. In the currently study the die-off rates of endogenous *E. coli* under field conditions was lower than those of STEC and *Salmonella*. The result would suggest that E coli would be a suitable surrogate for predicting the die off of *Salmonella* and STEC in the environment. However, the opposite was true under laboratory conditions.

Clostridium difficile endospores persisted over extended time periods although it was noted that ribotype germinated then subsequently declined over time.

Summary of Findings and Recommendations

The study determined the die off rates of STEC and Salmonella in manure (dairy and swine) amended soil (loam and sandy loam). The inoculated manure and soil mix was placed into vials that were placed at depth (15 cm) or on the surface of test plots. The trials were performed in the Spring (May) and Fall (October) with the temperature and soil moisture being monitored over the trial period. It was found that the die-off rates of STEC and Salmonella were variable so could not be fitted to linear or non-linear models. As a consequence the time for the population to decrease by 2 log cfu. By doing so it was found that the persistence of Salmonella was lower than that of STEC. Highest persistence of both pathogens was observed in loam soil at depth with lowest persistence on the surface of sandy soil. The rate of die-off of endogenous E. coli was lower than that of pathogens thereby suggesting it is a suitable metric for gauging the microbiological stats of soil. Although there was a relatively rapid die-off rates of STEC and Salmonella under field conditions persistent, low levels of sub-populations were observed over the trial period (90-120 days). Under laboratory conditions the die off rates STEC and Salmonella were lower compared to those observed in the field. Here, high temperature and moisture increase the die-off rate of Salmonella although STEC were less stable under low moisture. Generic *E. coli* exhibited higher die off rates in laboratory microcosms compared to STEC or *Salmonella*.
The persistence of *Clostridium difficile* endospores was ribotype dependent with 027 being more persistent compared to 078 which is commonly found in the environment.

Recommendations

The study has demonstrated that Salmonella and STEC can persist over 120 days in certain soil types. Consequently, it possible that pathogens could be present even after the 90 day wait period before planting crops. However, it should be noted that majority of the cells within the bacterial population die-off within the initial 14 day period leaving a small residual population that persists over an extended time.

Recommendation 1: Risk analysis should be performed on the hazard associated with low residual levels of pathogens with respect to contaminating crops or water courses.

Recommendation 2: The physiology of sub-populations of pathogens (i.e. STEC and Salmonella) should be studied to determine is environmental fitness has been acquired at the cost of virulence.

Recommendation 3: Algorithms should be developed based on predicting the die-off of pathogens under varying conditions of pH, temperature and soil type. The models developed can then predict the level of pathogen die-off under the measured climatic conditions. This would

provide a more accurate estimate of the wait period from manure application to planting crops than using the arbitrary 90 or 120 day rule.

Recommendation 4: Laboratory based soil microcosm models underestimate pathogen die off in the real environment and hence are suitable for risk assessment studies.

Recommendation 5: Selective methods for enumerating pathogens in soil and manure samples are required to be developed. This is especially the case for Listeria monocytogenes were selective, quantitative methods are lacking.

Recommendation 6: Further trials are required to verify that die-off models are applicable in different geographical areas. If not then further additional factors that contribute to the rate of pathogen die-off should be investigated.

APPENDICES

Publications and Presentations (required)

Food Safety Symposium Series
Poster Presentations
IAFP Annual International Association of Food Protection (Aug 2014)
OFPA Food Protection Spring Meeting (April 2014)
12th Food Safety Research Forum

Budget Summary (required)

To be submitted

Tables and Figures (optional)

Table 1: Pathogens, enumeration and enrichment methods used in the study.

Pathogen	**Isolation Source**	**Enumeration Method**	**Enrichment**
E coli O157:H7 ph3	Clinical	10g sample was suspended in 90 ml TSB + novobiocin. Plating was performed on CT-SMac and CHROMagar both of which were incubated at 37°C. Confirmation of presumptive positives was performed using RT-PCR	In the event of no colonies being recovered on plates the homogenate in TSBn was enriched for 24h at 37°C. The enriched sample was spread plated onto CR-SMac and CHROM agar which was incubated at 37°C for 24h. Presumptive positive was confirmed by PCR.
E coli O157:H7 C420	Manure		
E coli O26 AN02	Carcass		
E coli O145 AN 320	Carcass		
S Typhimurium	Pig	10 g sample was suspended in 90 ml in 0.1% peptone water. The dilution series was plated onto XLT-4 plates incubated at 37°C for 24 h. Presumptive colonies were confirmed using latex immune assay.	The homogenate was incubated at 37°C for 24h and aliquots (0.1ml) plated onto semi-solid RV medium that was incubated at 42°C for 16h. Presumptive positive colonies were streaked out onto XLT-4 that was incubated at 37°C for 24h. Presumptive colonies were confirmed using latex agglutination test.
S Montevideo	Tomato		
S Newport	Clinical		
S Enteritidis	Poultry		

L monocytogenes FL1	Clinical	10g sample was suspended in 90 ml one-step enrichment broth and homogenated. A dilution series was prepared and spread plated on Modified Oxford agar that was incubated at 30°C for 48h. Presumptive positive colonies were confirmed using latex agglutination test.	The homogenated sample was incubated at 37°C for 24h and subsequently streaked onto MOX. The plates were incubated at 30°C fror 48 h and positive colonies were confirmed using latex agglutination test.
L monocytogenes G438	Processing plant		
L monocytogenes FLA2	Soil		
L monocytogenes 1442	Clinical		
C difficile 027	Clinical	10 g of sample was placed into *C. difficile* moxalactam norfloxacin (CDMN) enrichment broth and homoagenized within an anaerobic cabinet. A dilution series was prepared and planted onto CDMN agar that was incubated at 37°C for 48h. Presumptive colonies were confirmed using a L-proline aminopeptidase assay.	The homogenate was incubated for 7 days at 37°C. Aliquots (1ml) of the enriched culture was mixed with 2 ml of absolute ethanol and incubated at room temperature for 1h. The mixture was centrifuged for 10 min at the speed of 4, 500 ×g; sediment was streaked onto CDMN agar and incubated anaerobically at 37°C for 48 h. Presumptive colonies were confirmed using the L-proline aminopeptidase assay and screened for heamolytic activity using 5% blood agar 48 hours at 37°C
C difficile 078	Pig manure		

Table 2: Bacteria inoculated into the different vial sets that were implanted into the different field plots used in the study.

Vial Set	**Bacteria inoculated into Soil:Manure mix**
1	STEC
2	*Salmonella* and *Listeria*
3	*Clostridium difficile* 027

4	*Clostridium difficile* 078
5	None (Control)

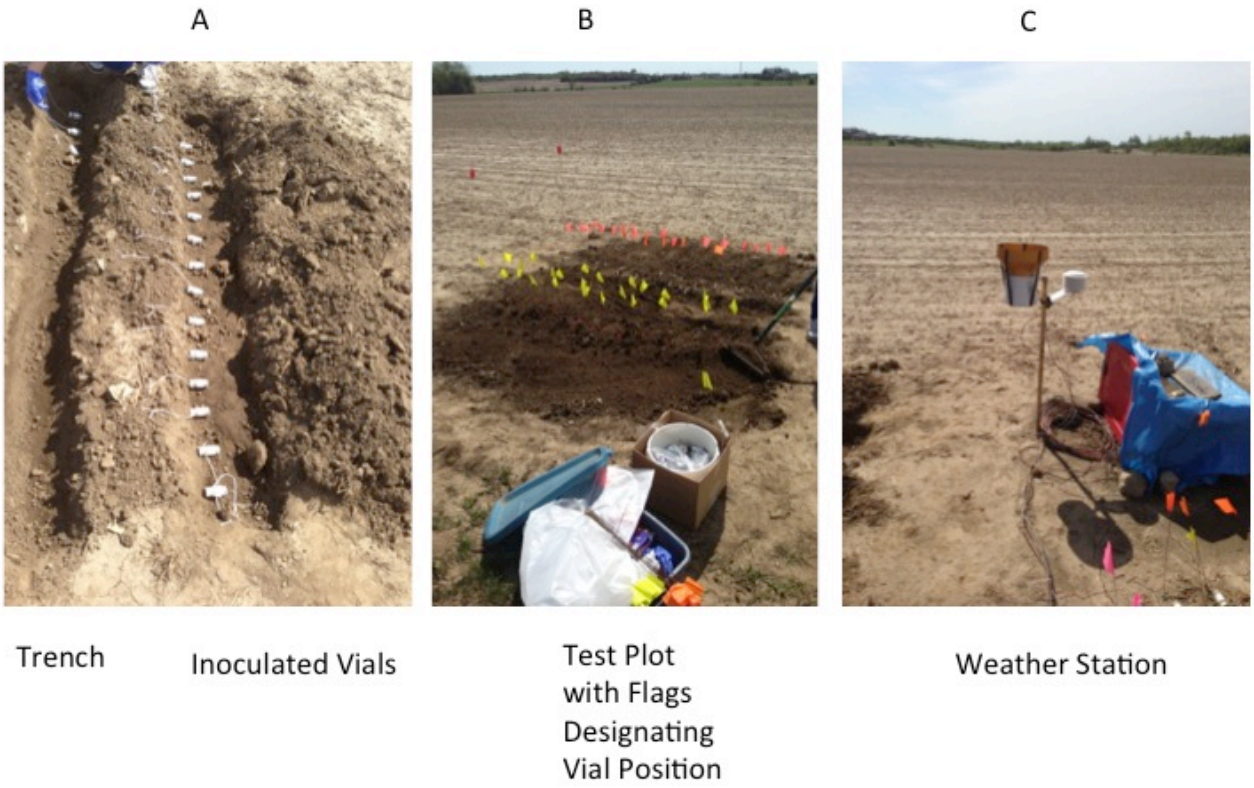


Figure 1: Images of one test plot used in the study. A trench was prepared into which the inoculated vials and controls were placed (A). The trenches were filled with the corresponding soil and a further set of vials placed on the surface (B). The temperature and rainfall were constantly measured using a remote weather stationed linked to a recorder

Table 3: Time for pathogens (STEC and *Salmonella*) and generic *E. coli* to decrease by 2 log or 3 log cfu/g from the original inoculation level. The test pathogen was inoculated into manure then into soil prior to placing into vials that were subsequently planted at depth or on the surface of the field test plot. Vials were withdrawn throughout the 90 – 120 day test period for enumeration of survivors.

STEC/ Trial	**Manure Type**	**Sandy (Days)**				**Sandy Loam (Days)**			
		Surface		**Depth**		**Surface**		**Depth**	
		2 LCR	**3 LCR**	**2 LCR**	**3 LCR**	**2 LCR**	**3 LCR**	**2 LCR**	**3 LCR**
Spring 1	Dairy	2	5	48	52	2	5	48	>90
Fall	Dairy	2	8	2	5	2	7	12	>120
Spring 2	Dairy	2	5	8	12	2	5	23	37
Spring 1	Swine	2	4	4	4	2	6	3	4
Fall	Swine	2	5	10	68	8	10	12	32
Spring 2	Swine	5	18	8	12	3	4	5	48

LCR: Log count reduction

Salmo	**Manure**	**Sandy (Days)**	**Sandy Loam (Days)**

Keith Warriner, University of Guelph

Die-off rates of human pathogens in manure amended soil under natural climatic conditions using novel sentinel chamber system

nella	**Type**	**Surface**		**Depth**		**Surface**		**Depth**	
		2 LCR	**3 LCR**	**2 LCR**	**3 LCR**	**2 LCR**	**3 LCR**	**2 LCR**	**3 LCR**
Spring 1	Dairy	2	4	4	5	2	5	29	>120
Fall	Dairy	2	4	3	5	2	7	29	>120
Spring 2	Dairy	4	12	7	12	4	8	21	39
Spring 1	Swine	10	48	20	25	48	38	56	>120
Fall	Swine	2	5	28	68	5	28	22	28
Spring 2	Swine	7	12	9	13	4	7	20	42

***E coli**		**Sandy (Days)**		**Sandy Loam (Days)**	
	Manure Type	**Surface**	**Depth**	**Surface**	**Depth**
		2 LCR	**2 LCR**	**2 LCR**	**2 LCR**
Spring 1	**Dairy**	**20**	**36**	**20**	**>90**
Fall	**Dairy**	**128**	**32**	**5**	**96**
Spring 2	**Dairy**	**1**	**4**	**5**	**39**
Spring 1	**Swine**	**2**	**32**	**2**	**>90**
Fall	**Swine**				
Spring 2	**Swine**	**9**	**9**	**19**	**9**

Endogenous non-pathogenic *E. coli.

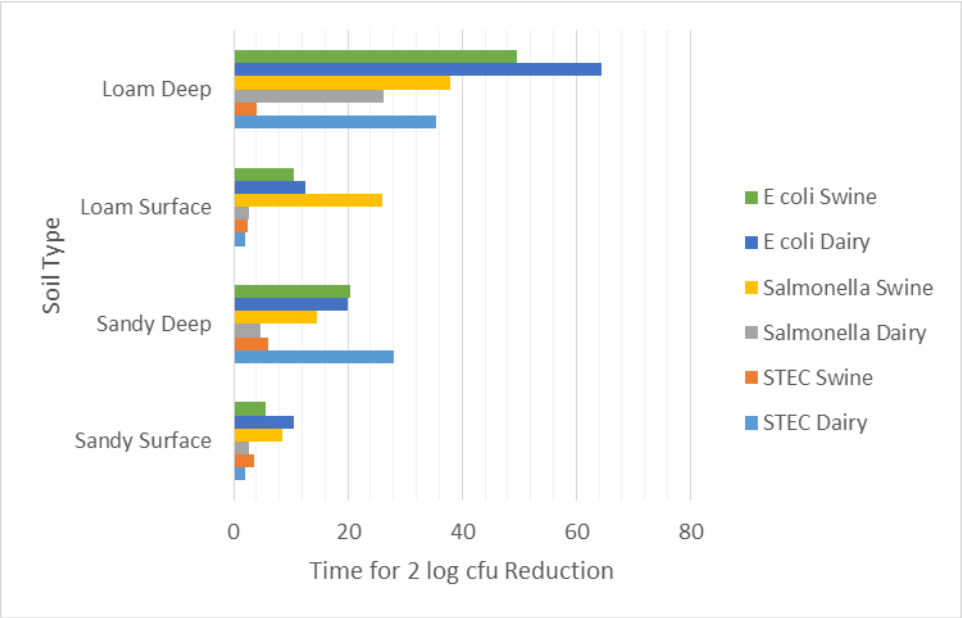


Figure 2: Time to achieve a 2 log cfu/g die off in STEC and Salmonella in different soil and manure types. The time to achieve a 2 log cfu/g reduction was taken from trials performed in Spring 2013 and 2014.

Table 4: Die-off rate of STEC, Salmonella and E. coli in laboratory manure (dairy) amended sandy loam soil. Microcosoms (3 liter volume) of soil amended manure was inoculated with Salmonella or STEC cocktail to a final density (5 log cfu/g). The microcosoms were then placed in environmental chambers under defined temperature and relative humidity. Core samples of soil were taken for enumeration of *E. coli*, STEC or *Salmonella*. Bacterial levels were plotted and the linear part of the curve used to calculate the inactivation rate constant.

Temperature	**Moisture (%)**	***Salmonella***		**STEC**		**Generic *E coli***	
		r^2	***k***	**r^2**	***k***	**r^2**	***k***
4°C	5	0.259	0.077	0.184	0.11	0.866	0.102
10°C	5	0.398	0.116	0.14	0.044	0.755	0.106
20°C	5	0.864	0.15	0.34	0.114	0.866	0.102
30°C	5	0.958	0.116	0.95	0.55	0.965	0.231
4°C	15	0.263	0.08	0.148	0.12	0.601	0.109
10°C	15	0.6	0.15	0.272	0.157	0.928	0.106
20°C	15	0.5	0.144	0.63	0.176	0.936	0.173
30°C	15	0.559	0.164	810	0.523	0.755	0.144
4°C	25	0.318	0.109	0.26	0.159	0.365	0.046

10°C	25	Not Determined		0.082	0.041	0.956	0.0914
20°C	25	0.075	0.095	0.35	0.074	0.78	0.118
30°C	25	0.614	0.205	0.64	0.117	0.731	0.065

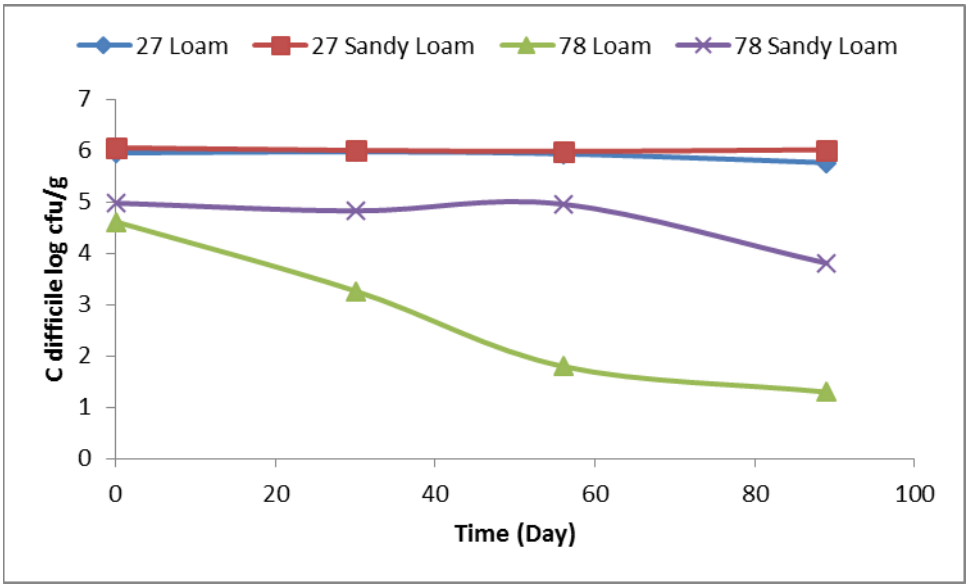


Figure 3: Levels of *Clostridium difficile* 027 and 078 spores in two different soil types amended with biosolids derived from sewage treatment works.

Suggestions to CPS (optional)

The PI and co-PI wish to extend their gratitude for the financial support for undertaking the project. We would also like to put forward a suggestion that a network of researchers should be setup to pool data from pathogen die-off studies to make more powerful predictive models in the future.

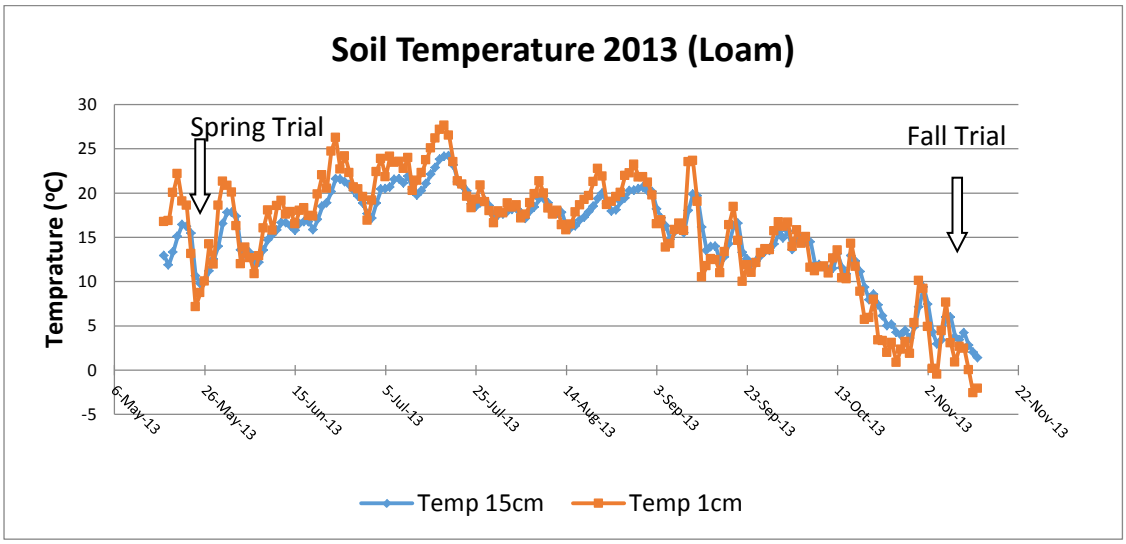
References

Avery, S.M., Moore, A. and Hutchison, M.L. (2004) Fate of Escherichia coli originating from livestock faeces deposited directly onto pasture. *Letters in Applied Microbiology* **38**, 355-359.
Bolton, D.J., Monaghan, A., Byrne, B., Fanning, S., Sweeney, T. and McDowell, D.A. (2011) Incidence and survival of non-O157 verocytotoxigenic Escherichia coli in soil. *Journal of Applied Microbiology* **111**, 484-490.
Duriez, P. and Topp, E. (2007) Temporal dynamics and impact of manure storage on antibiotic resistance patterns and population structure of Escherichia coli isolates from a commercial swine farm. *Applied and Environmental Microbiology* **73**, 5486-5493.
Eamens, G.J., Dorahy, C.J., Muirhead, L., Enman, B., Pengelly, P., Barchia, I.M., Gonsalves, J.R. and Cooper, K. (2011) Bacterial survival studies to assess the efficacy of static pile

composting and above ground burial for disposal of bovine carcases. *Journal of Applied Microbiology* **110**, 1402-1413.
Erickson, M.C., Habteselassie, M.Y., Liao, J., Webb, C.C., Mantripragada, V., Davey, L.E. and Doyle, M.P. (2014) Examination of factors for use as potential predictors of human enteric pathogen survival in soil. *Journal of Applied Microbiology* **116**, 335-349.
Fremaux, B., Prigent-Combaret, C., Beutin, L., Gleizal, A., Trevisan, D., Quetin, P., Jocteur-Monrozier, L. and Rozand, C. (2010) Survival and spread of Shiga toxin-producing Escherichia coli in alpine pasture grasslands. *Journal of Applied Microbiology* **108**, 1332-1343.
Fremaux, B., Prigent-Combaret, C. and Vernozy-Rozand, C. (2008) Long-term survival of Shiga toxin-producing Escherichia coli in cattle effluents and environment: An updated review. *Veterinary Microbiology* **132**, 1-18.
Holley, R.A., Arrus, K.M., Ominski, K.H., Tenuta, M. and Blank, G. (2006) Salmonella survival in manure-treated soils during simulated seasonal temperature exposure. *Journal of Environmental Quality* **35**, 1170-1180.
Hutchison, M.L., Walters, L.D., Moore, T., Thomas, D.J.I. and Avery, S.M. (2005) Fate of pathogens present in livestock wastes spread onto fescue plots. *Applied and Environmental Microbiology* **71**, 691-696.
Ibekwe, A.M., Ma, J.C., Crowley, D.E., Yang, C.H., Johnson, A.M., Petrossian, T.C. and Lum, P.Y. (2014) Topological data analysis of Escherichia coli O157:H7 and non-O157 survival in soils. *Frontiers in Cellular and Infection Microbiology* **4**, 11.
Ma, J., Ibekwe, A.M., Crowley, D.E. and Yang, C.-H. (2012) Persistence of Escherichia coli O157:H7 in Major Leafy Green Producing Soils. *Environmental Science & Technology* **46**, 12154-12161.
Robertson, L.J. and Gjerde, B.K. (2006) Fate of Cryptosporidium oocysts and Giardia cysts in the norwegian aquatic environment over winter. *Microbial Ecology* **52**, 597-602.
Scott, A., Conn, K.L., Lazarovits, G. and Topp, E. (2006) Dynamics of Escherichia coli in agricultural soils receiving swine manure slurry or liquid municipal biosolids. *Canadian Journal of Soil Science* **86**, 841-849.
Topp, E., Scott, A., Lapen, D.R., Lyautey, E. and Duriez, P. (2009) Livestock waste treatment systems for reducing environmental exposure to hazardous enteric pathogens: Some considerations. *Bioresource Technology* **100**, 5395-5398.
Warriner, K., Huber, A., Namvar, A., Fan, W. and Dunfield, K. (2009) Recent Advances in the Microbial Safety of Fresh Fruits and Vegetables. In *Advances in Food and Nutrition Research, Vol 57* ed. Taylor, S.L. pp.155-208: Elsevier Academic Press Inc, 525 B Street, Suite 1900, San Diego, Ca 92101-4495 USA.

Warriner, Keith
Results Annex A

Spring 2013 Field Trials



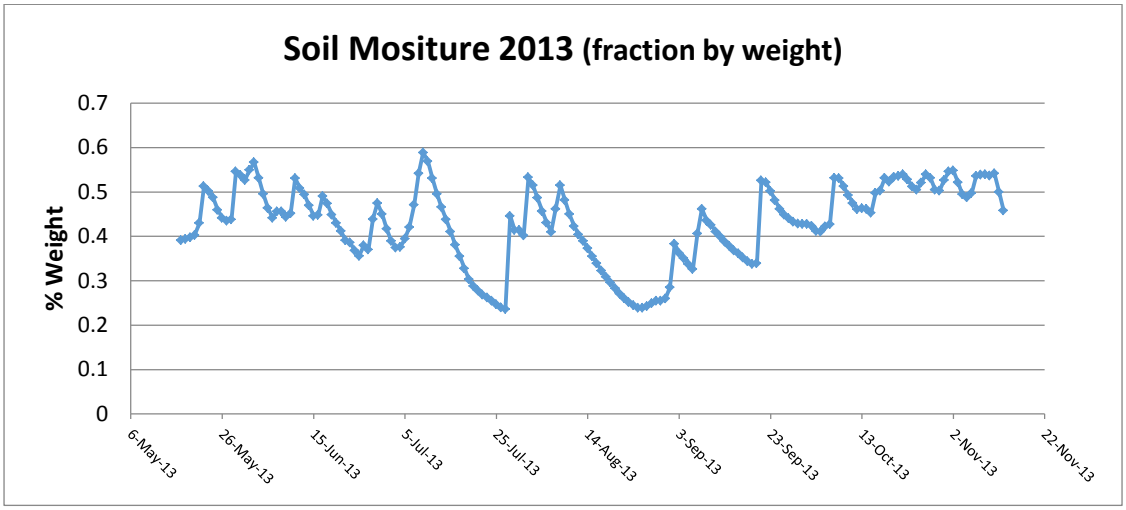


Figure 1. Soil temperature and moisture content of field plot throughout Spring Trial 1.

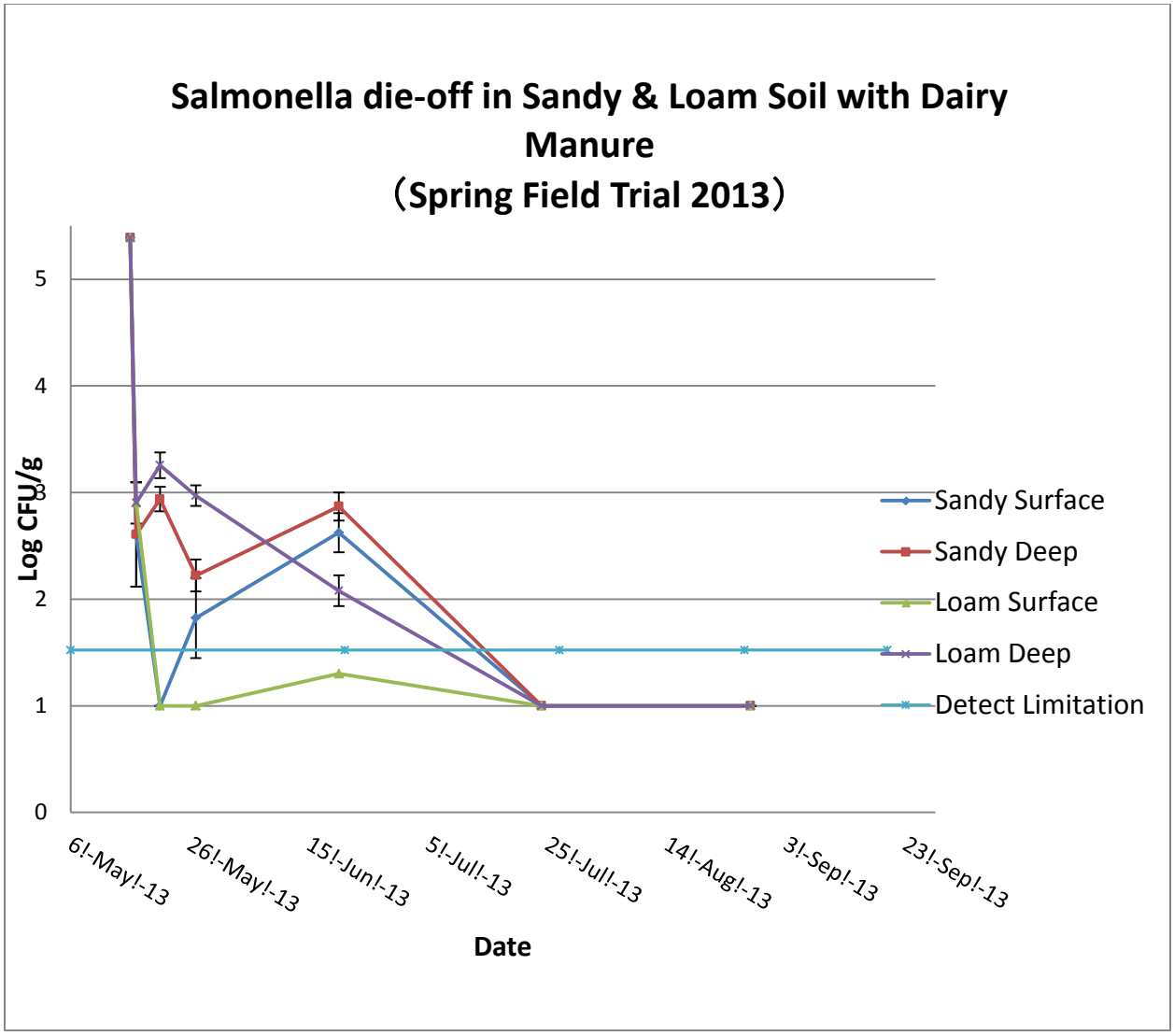


Figure 2: *Salmonella* levels in dairy manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

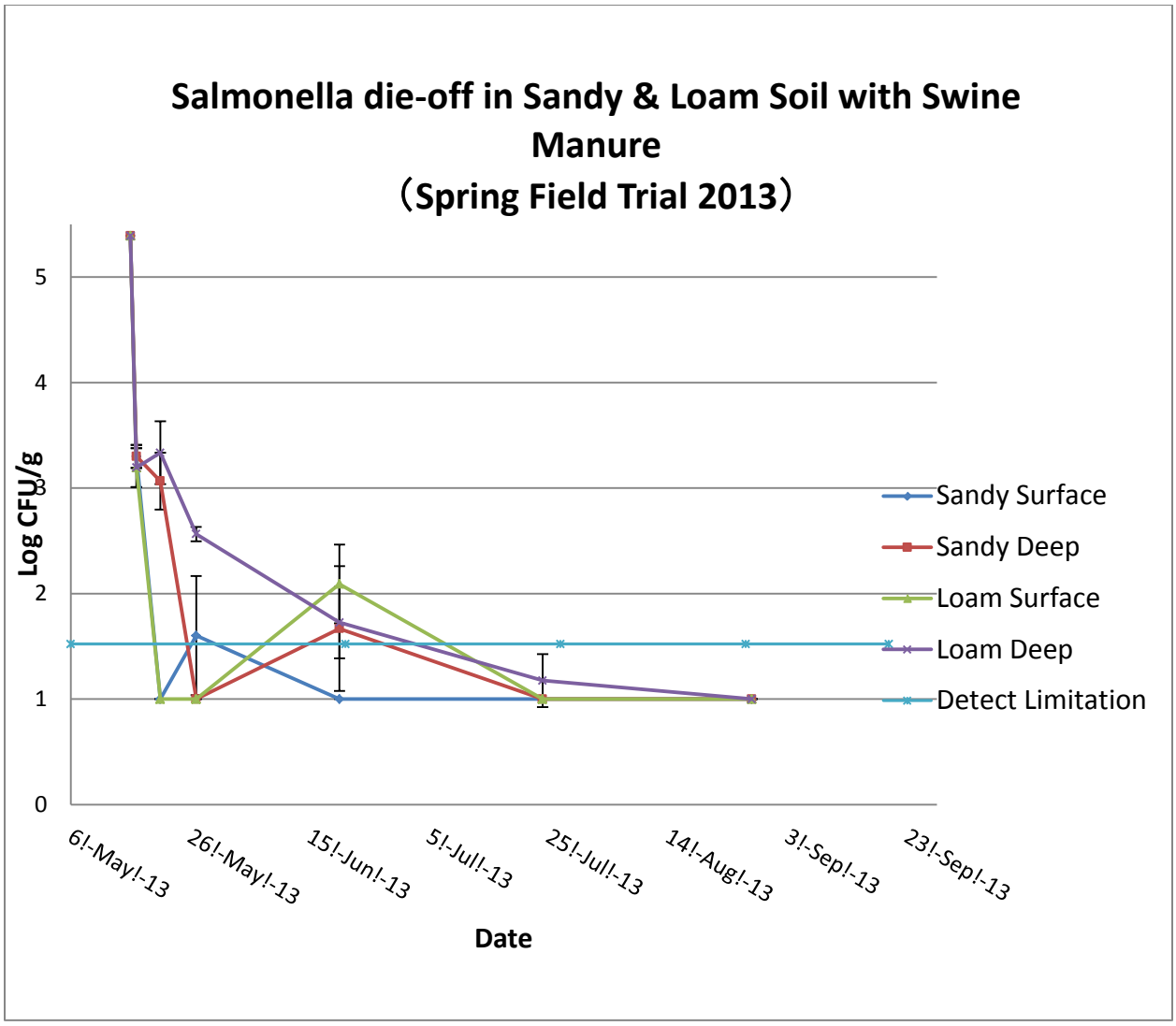


Figure 3: *Salmonella* levels in swine manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

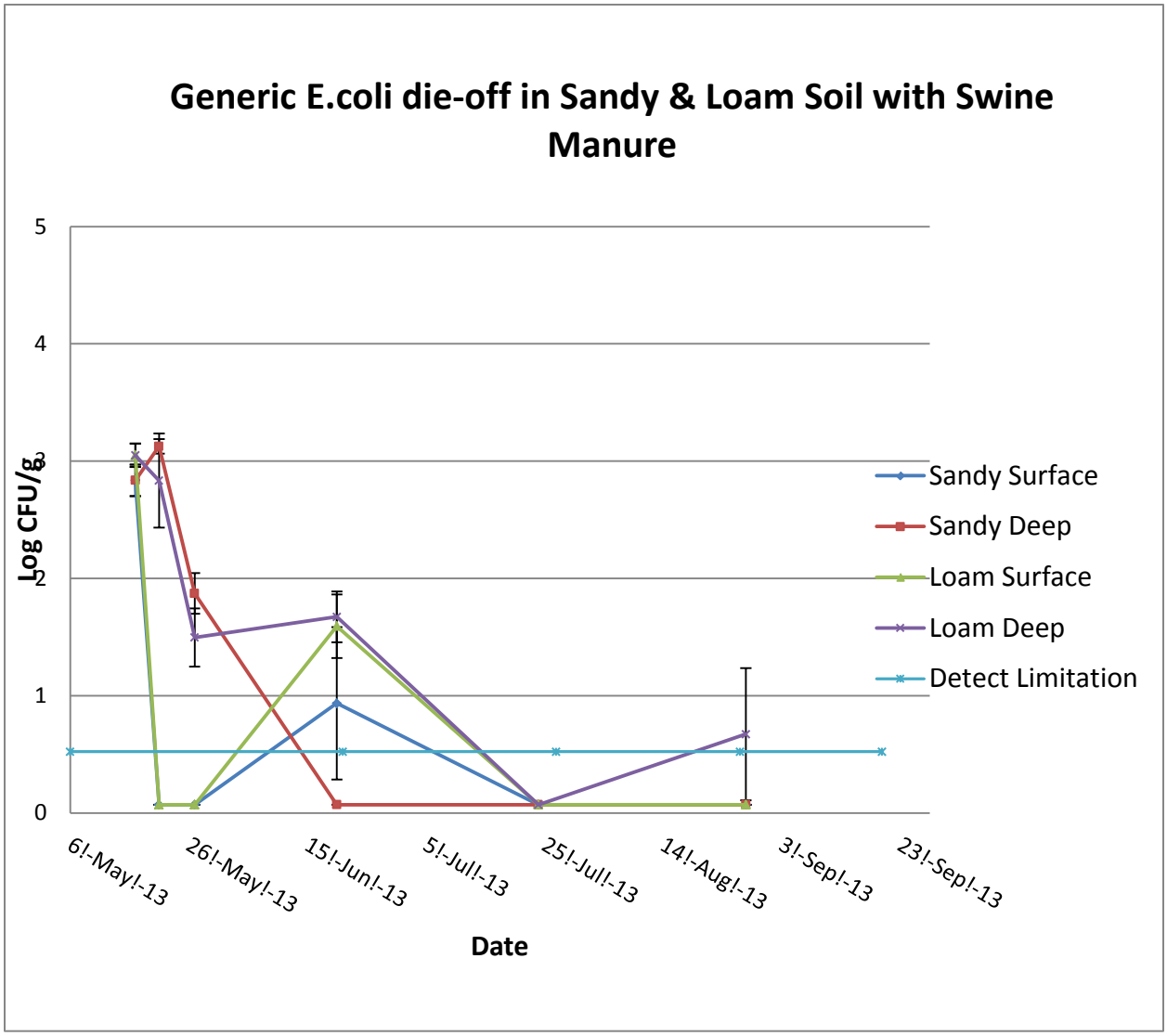


Figure 4: *Generic E. coli* levels in swine manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

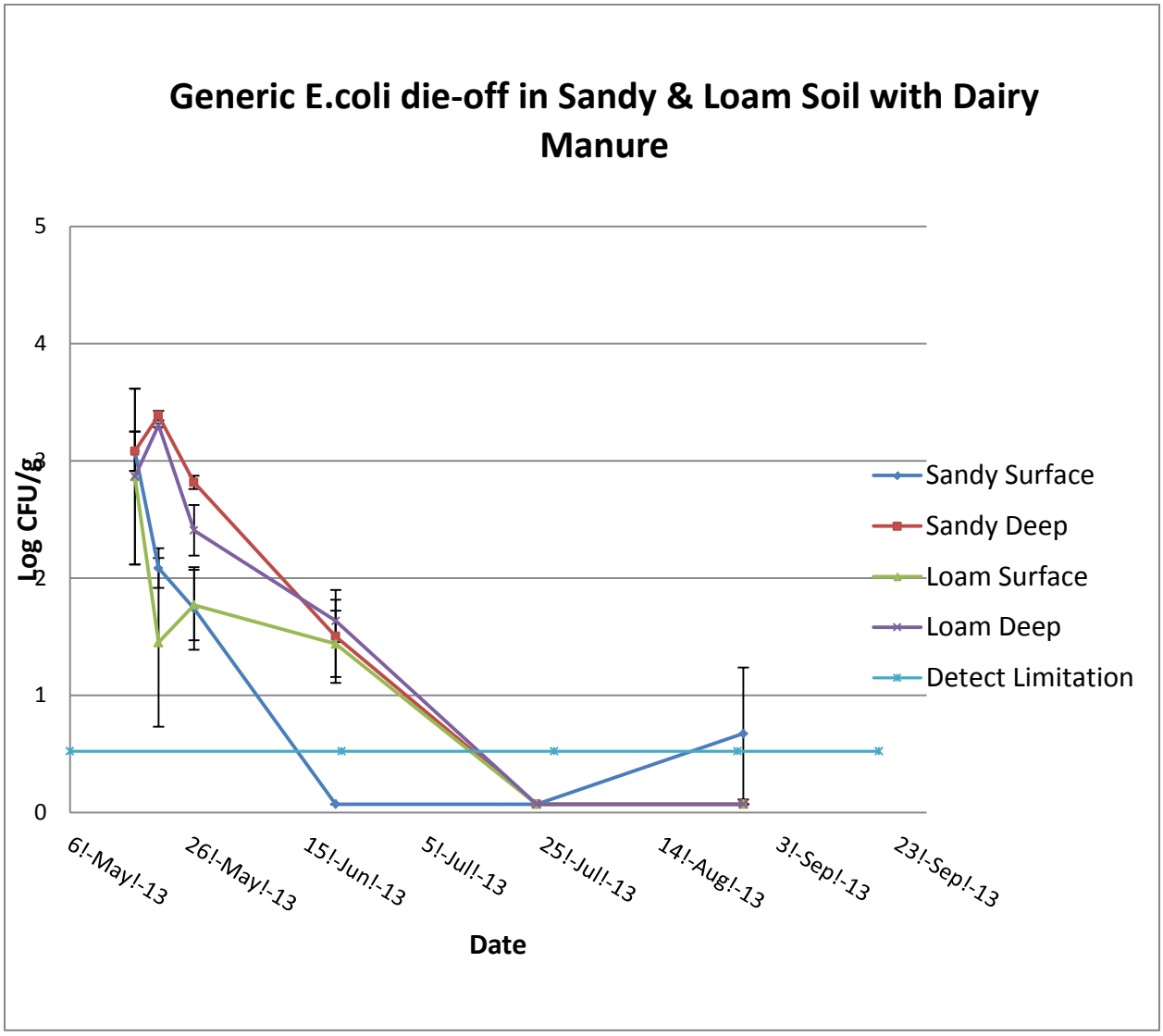


Figure 5: *Generic E. coli* levels in dairy manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

Fall 2013 Field Trials

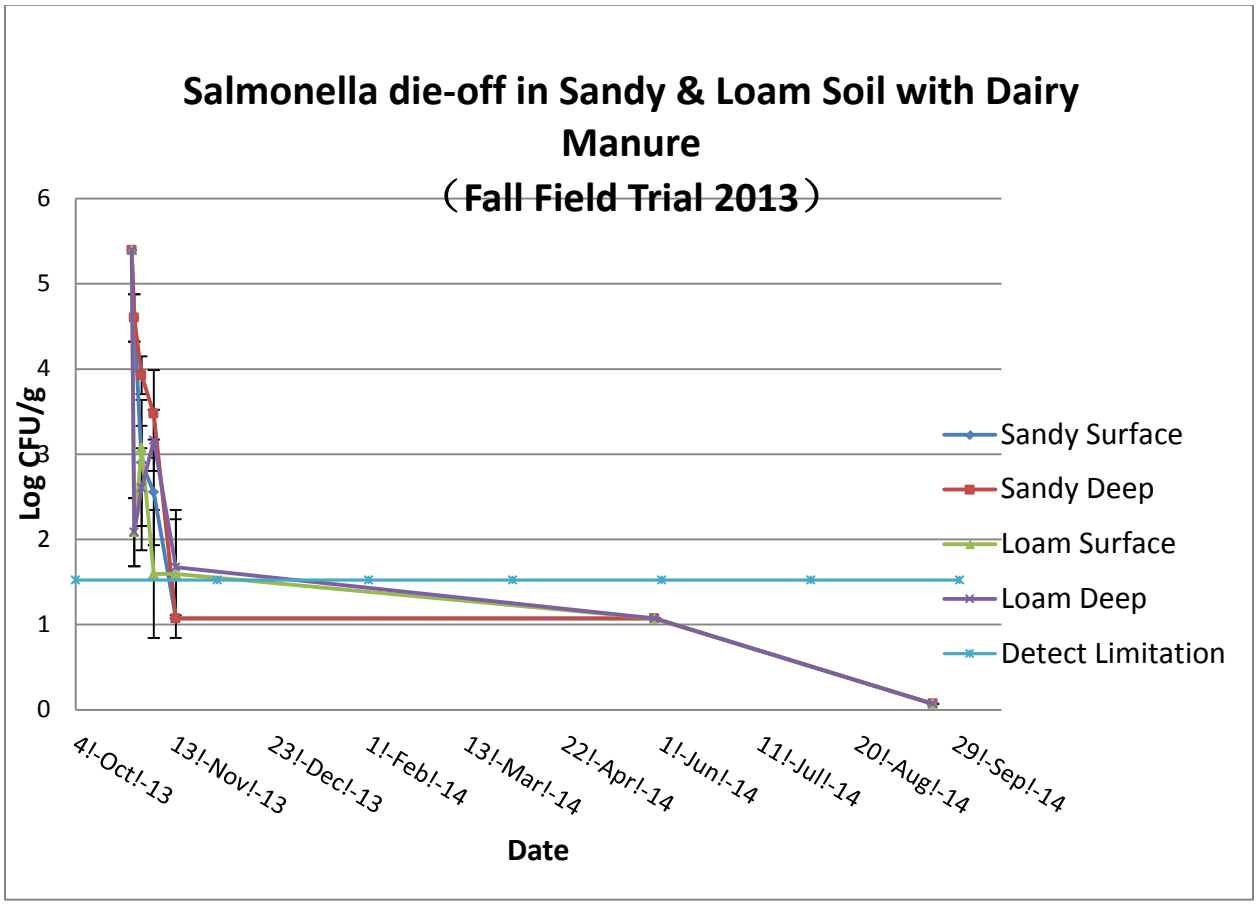


Figure 6: *Salmonella* levels in dairy manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

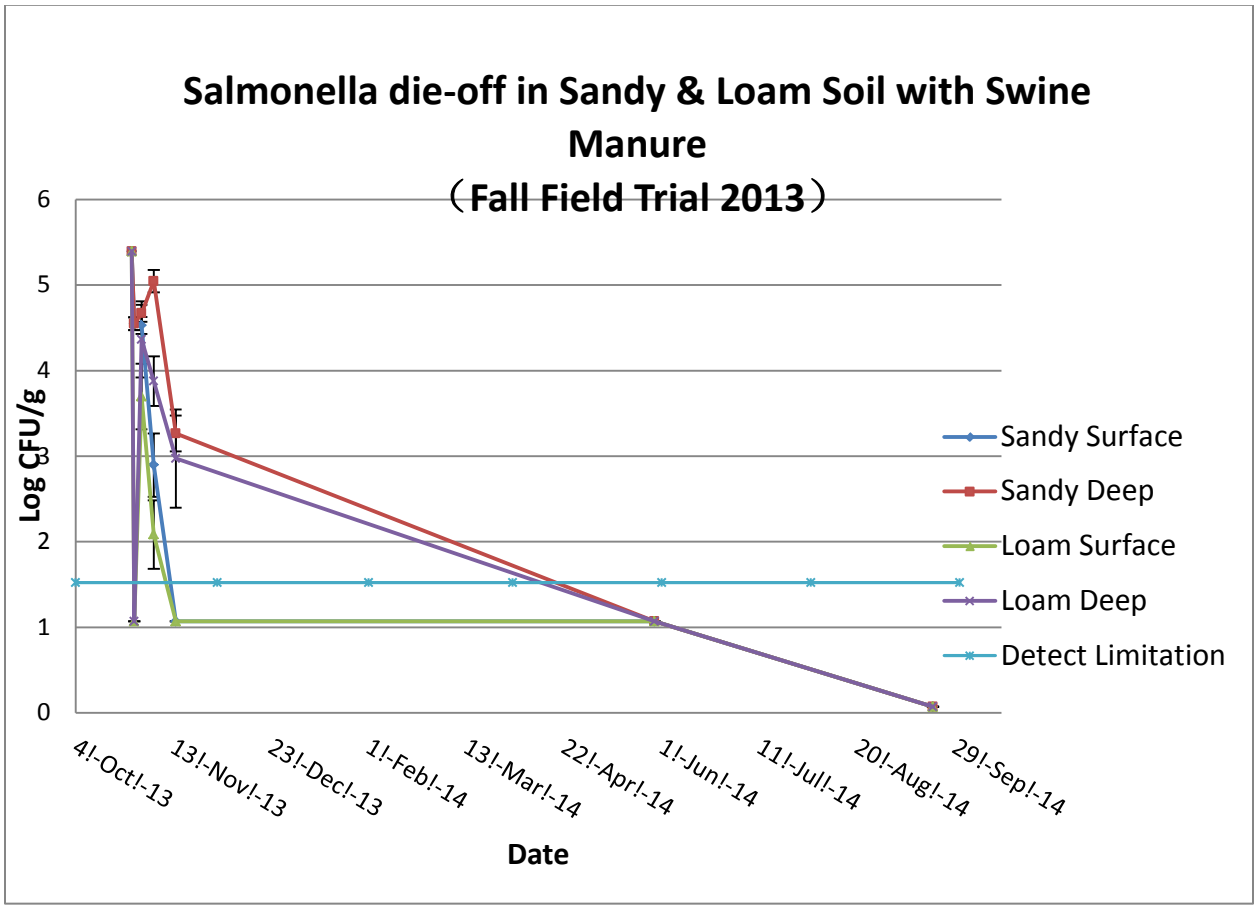


Figure 7: *Salmonella* levels in swine manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

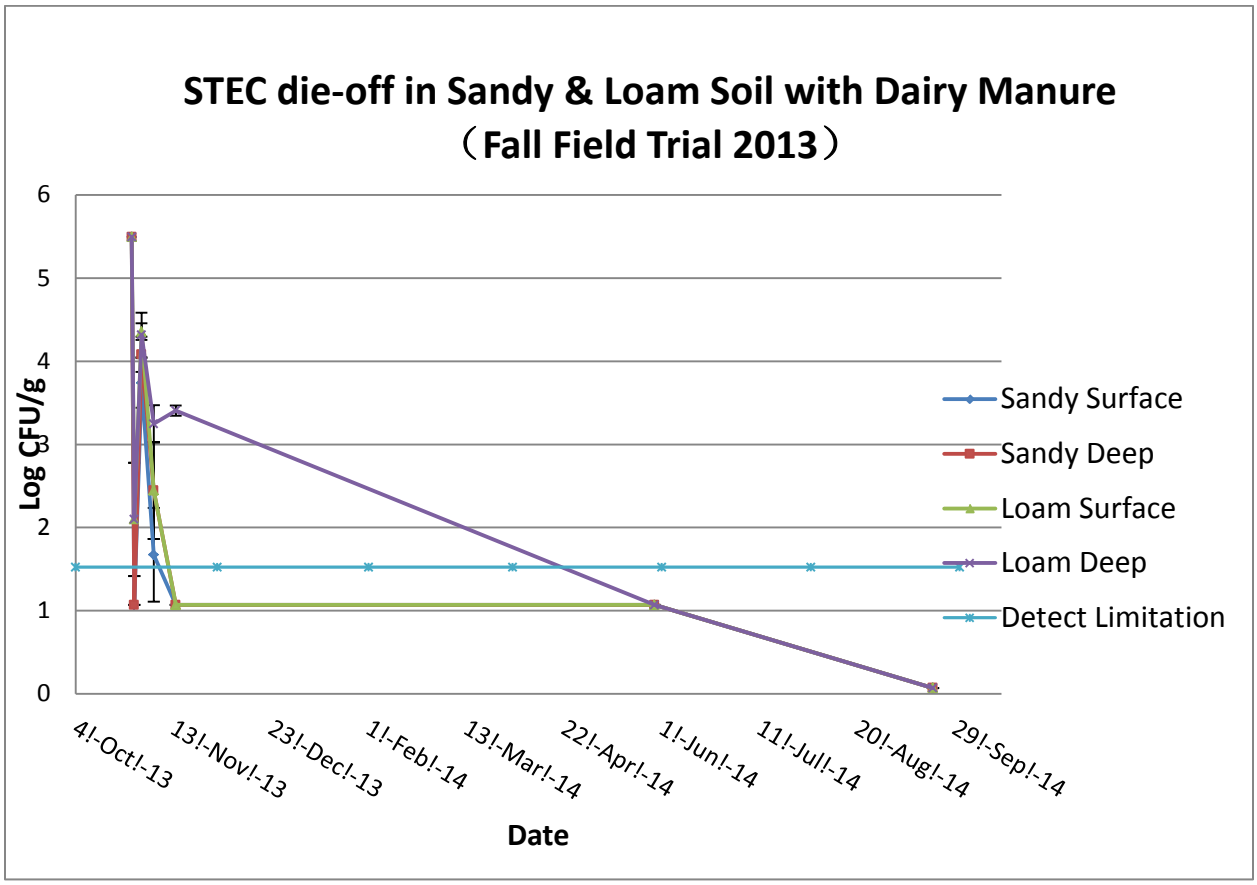


Figure 8: STEC levels in dairy manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

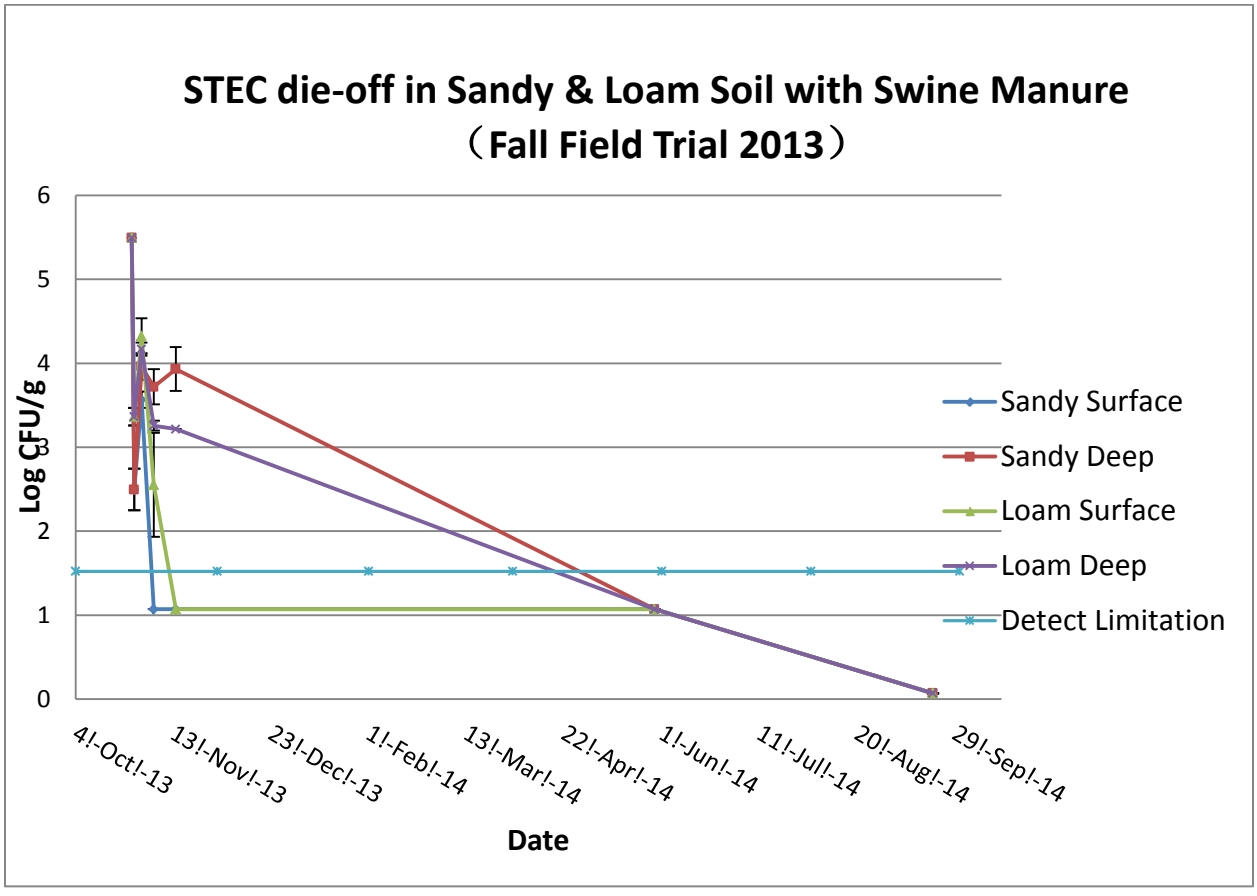


Figure 9: STEC levels in swine manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

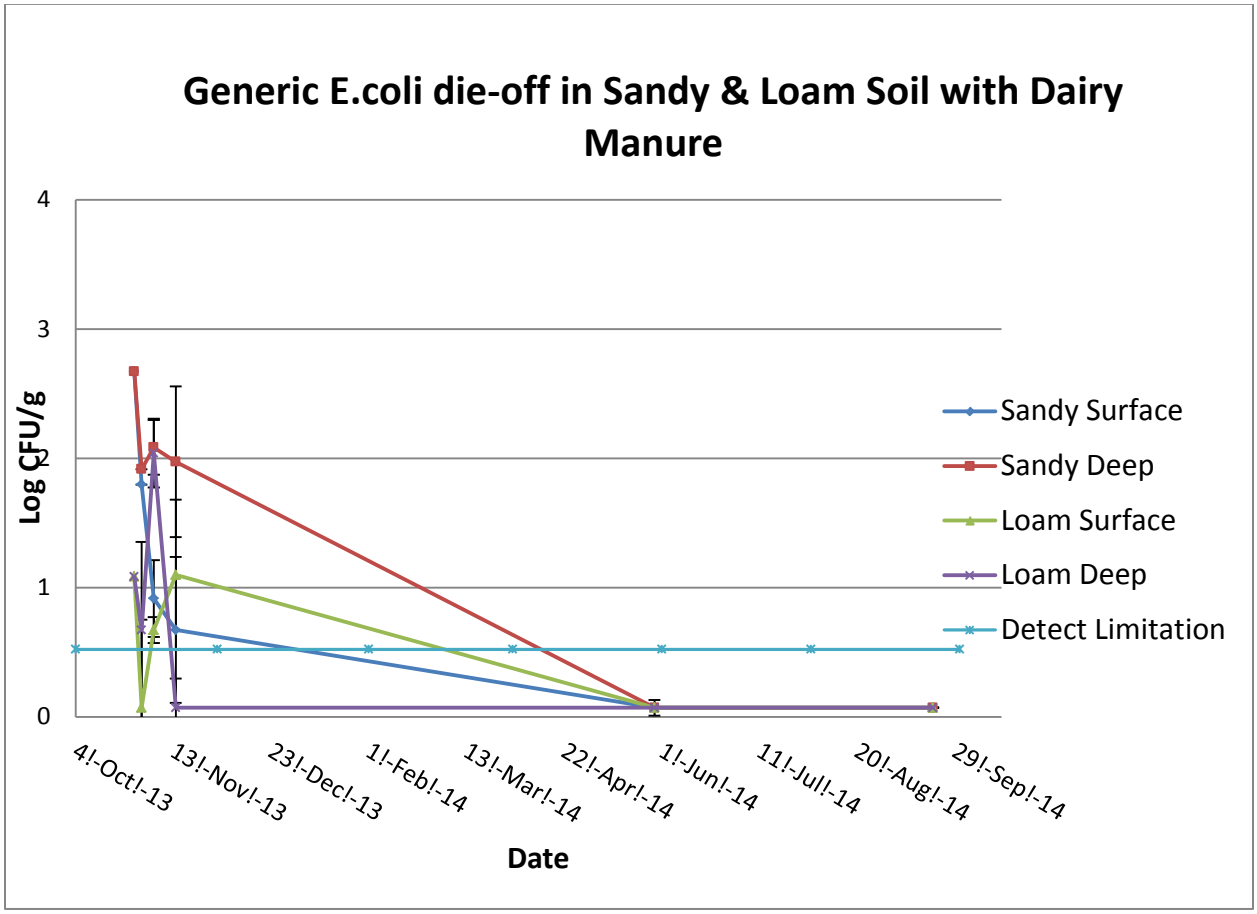


Figure 10: Generic *E. coli* levels in dairy manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

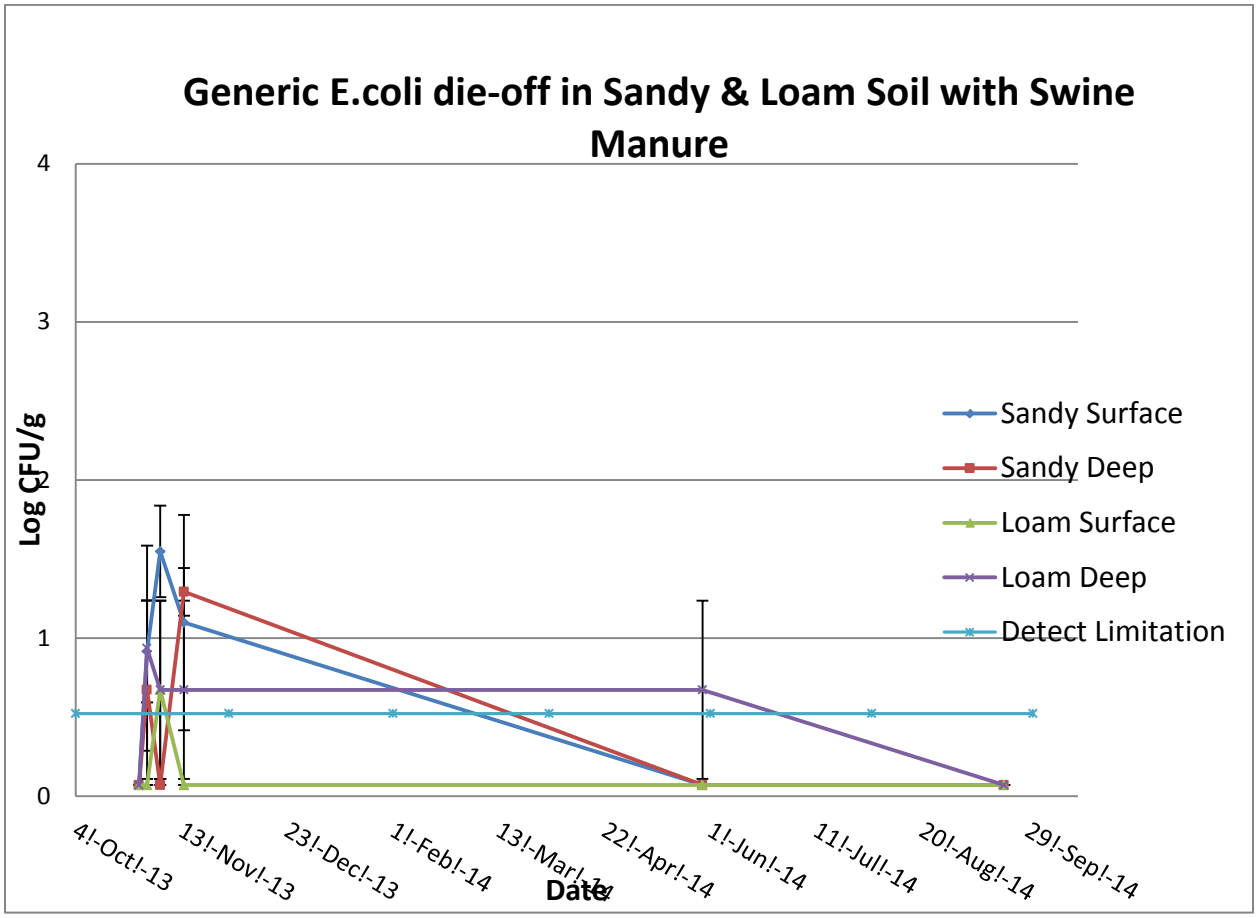
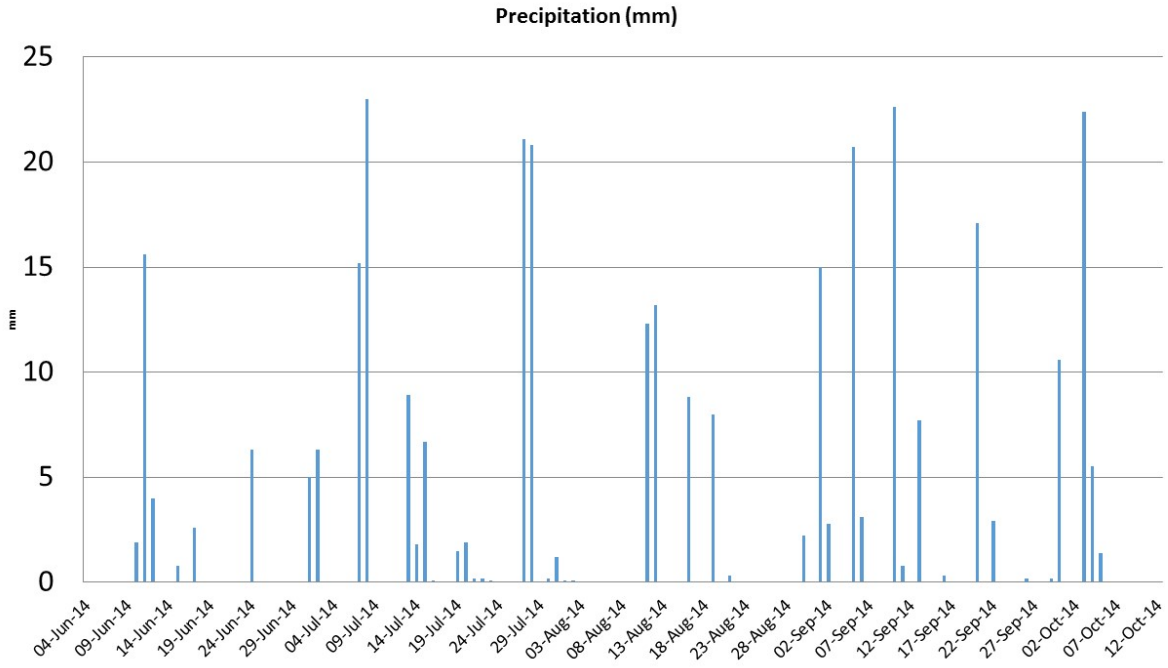
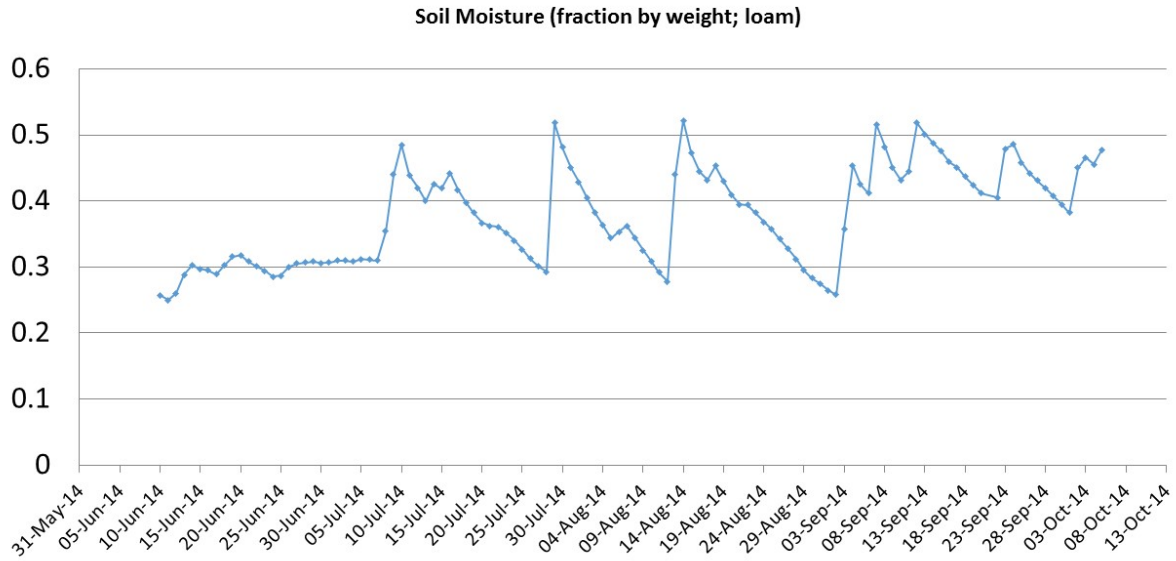
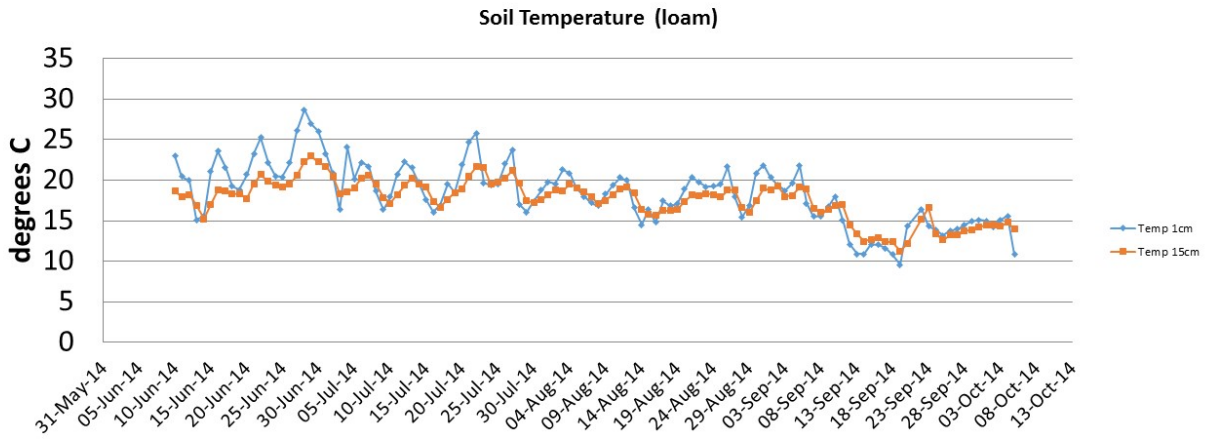


Figure 11: Generic *E. coli* levels in swine manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

Spring 2014 Field Trials







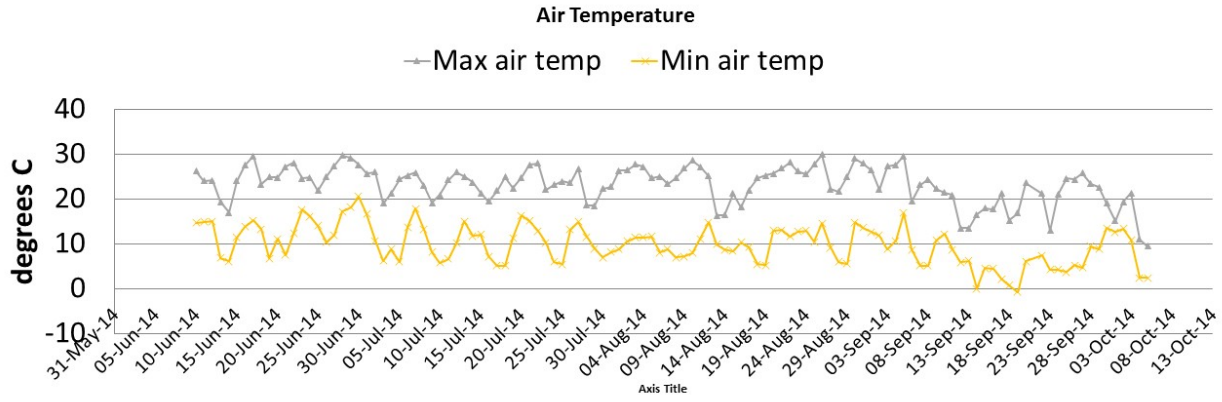


Figure 12: Environmental conditions throughout the Spring 2014 trial.

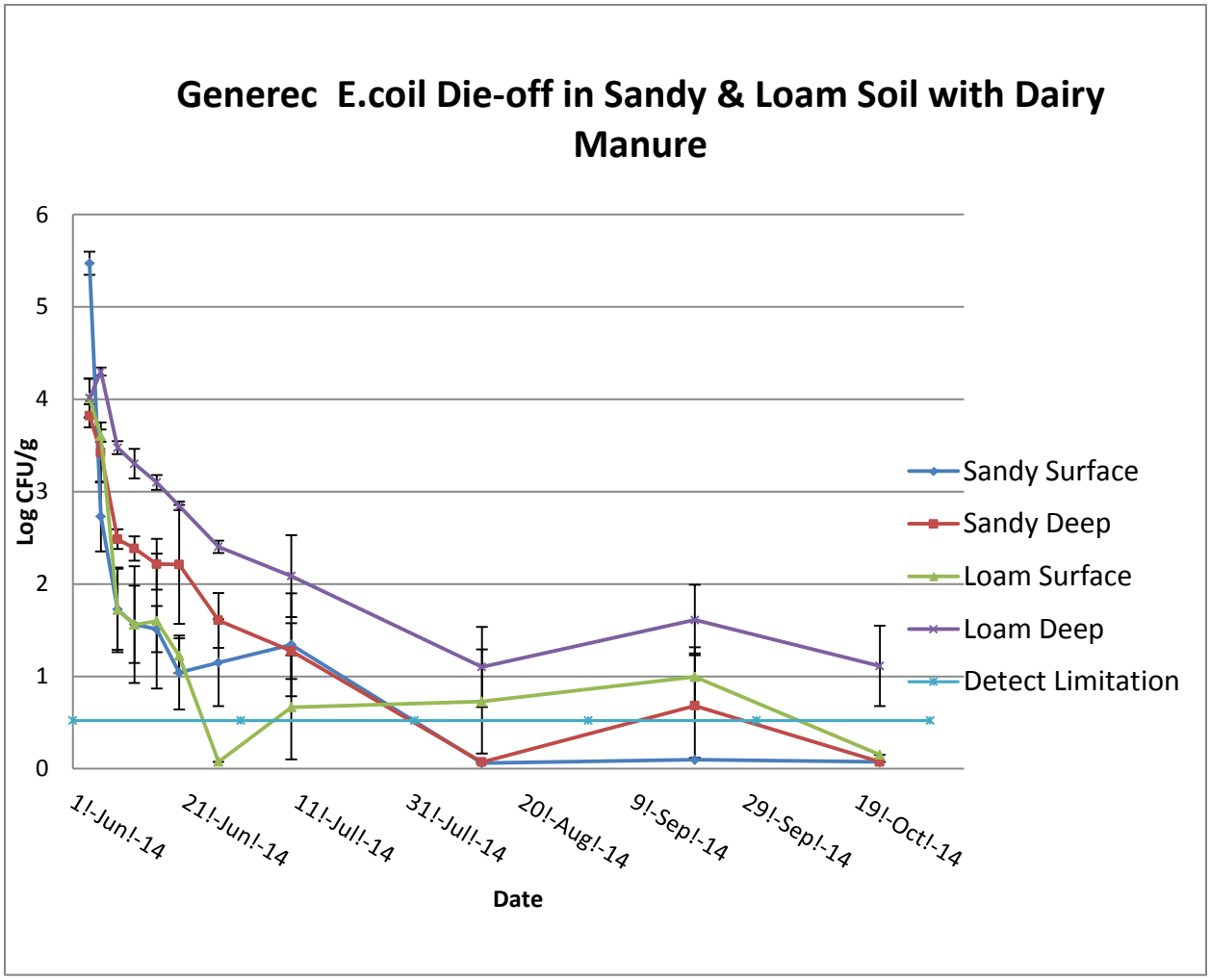


Figure 13: Generic *E. coli* levels in dairy manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

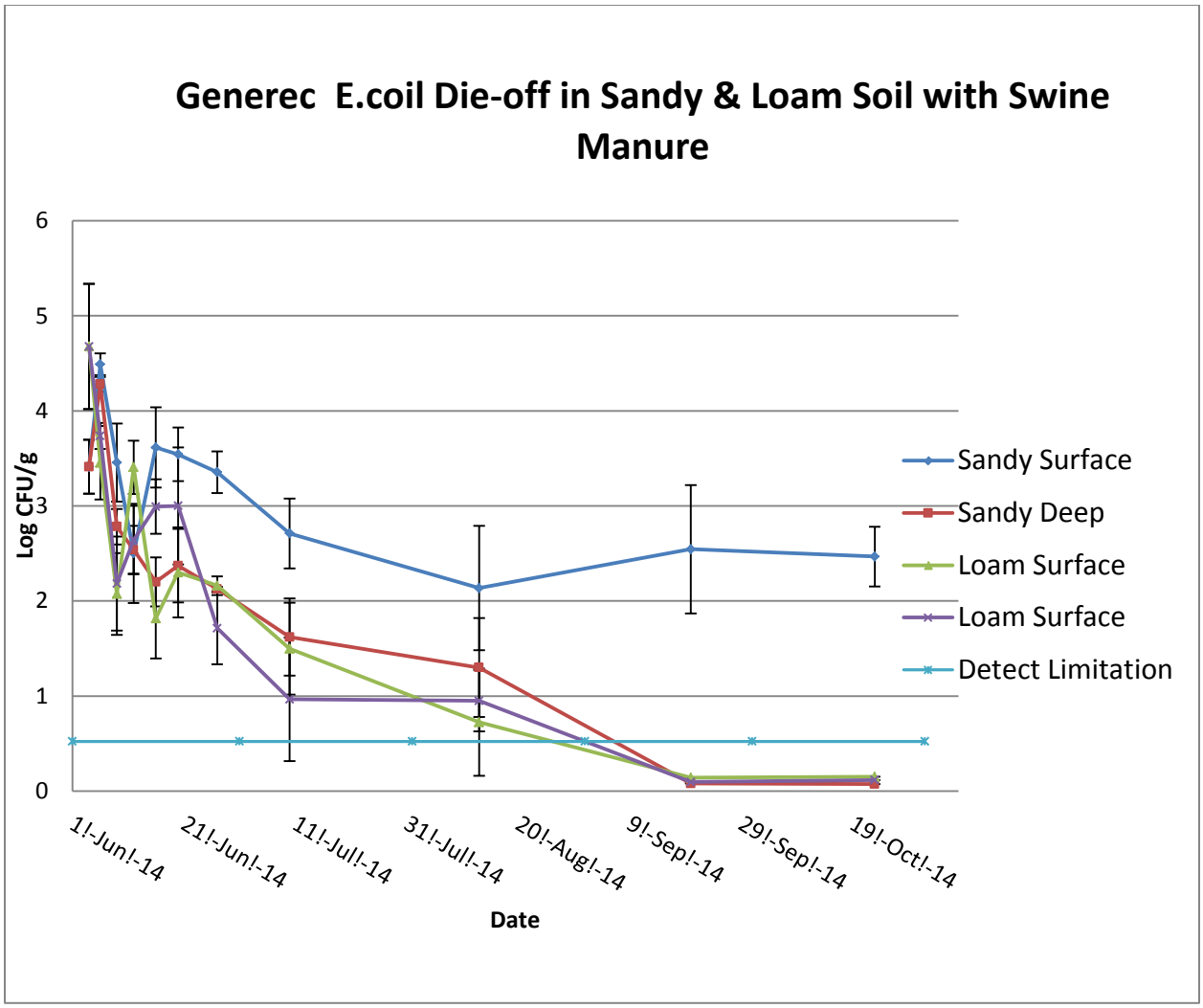


Figure 14: Generic *E. coli* levels in swine manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

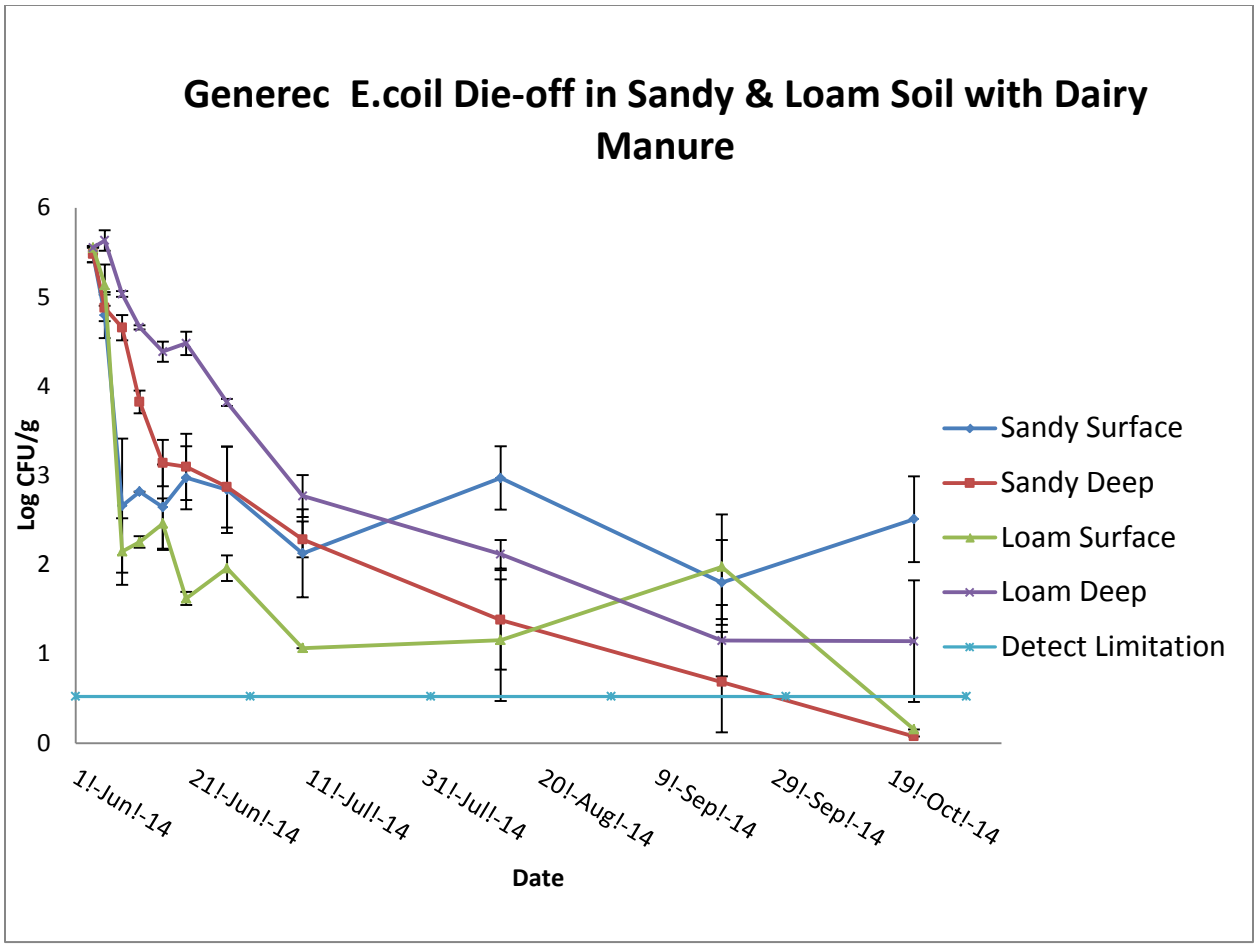


Figure 15: Generic *E. coli* levels in dairy manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

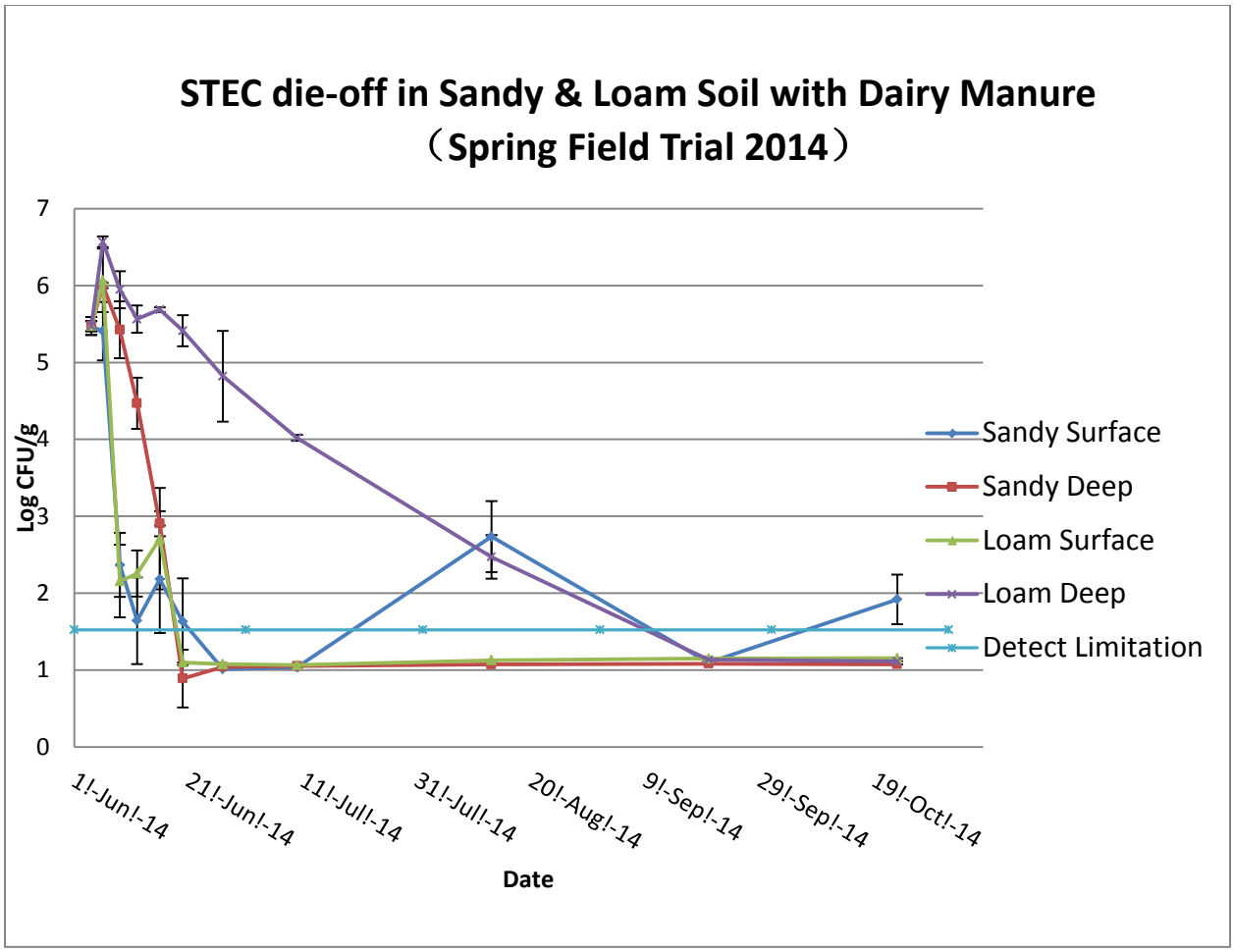


Figure 16: STEC levels in dairy manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

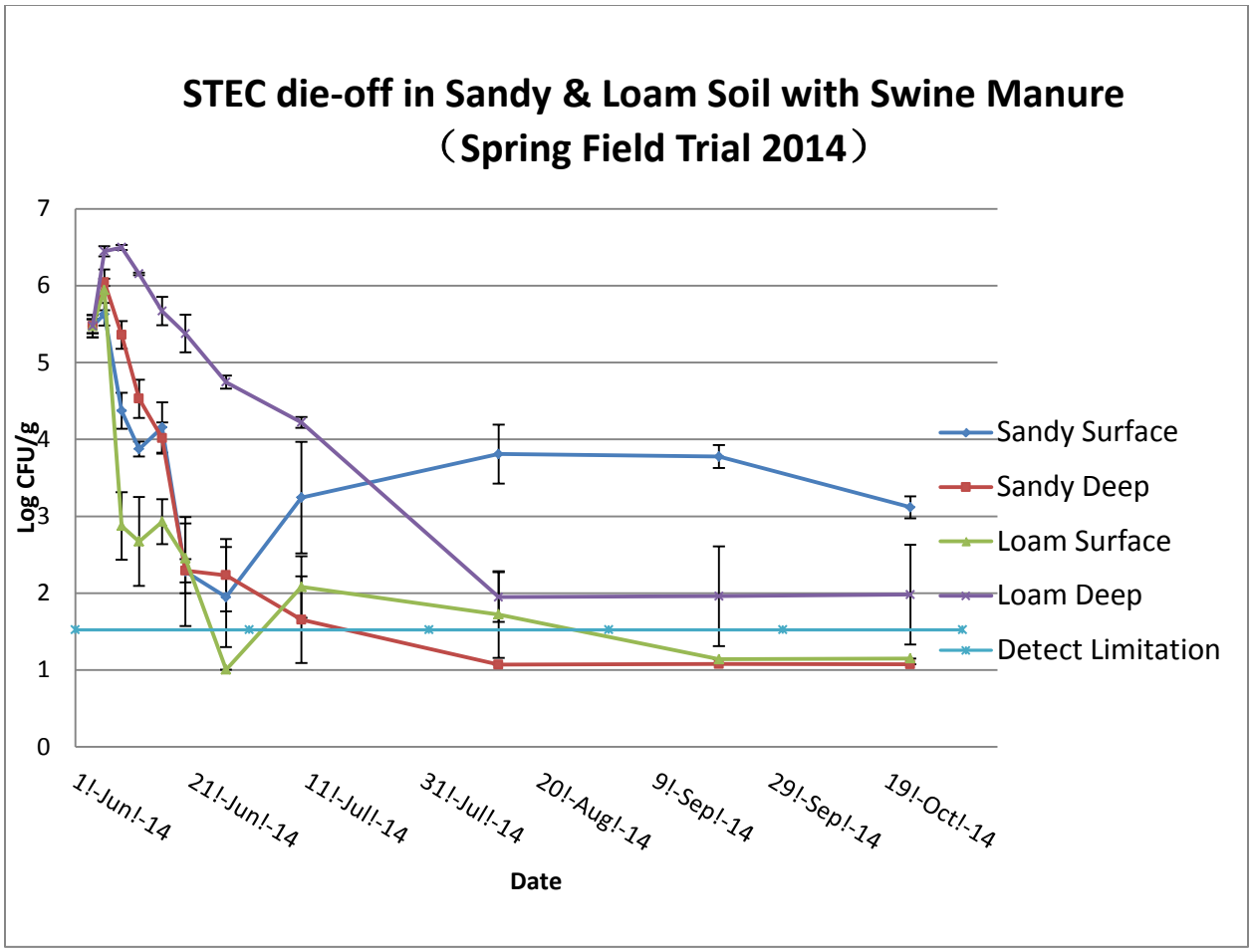


Figure 17: STEC levels in swine manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

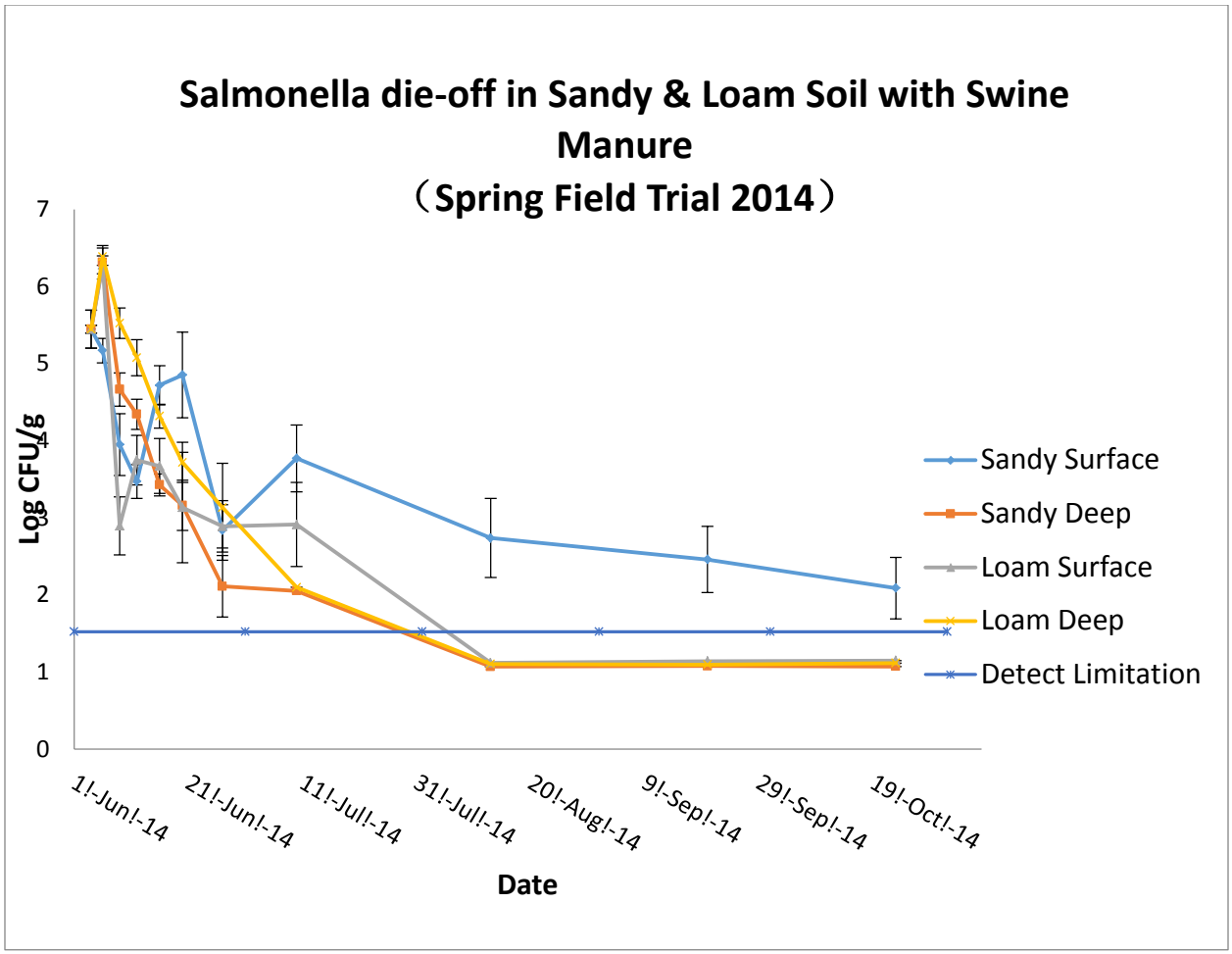


Figure 18: *Salmonella* levels in swine manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

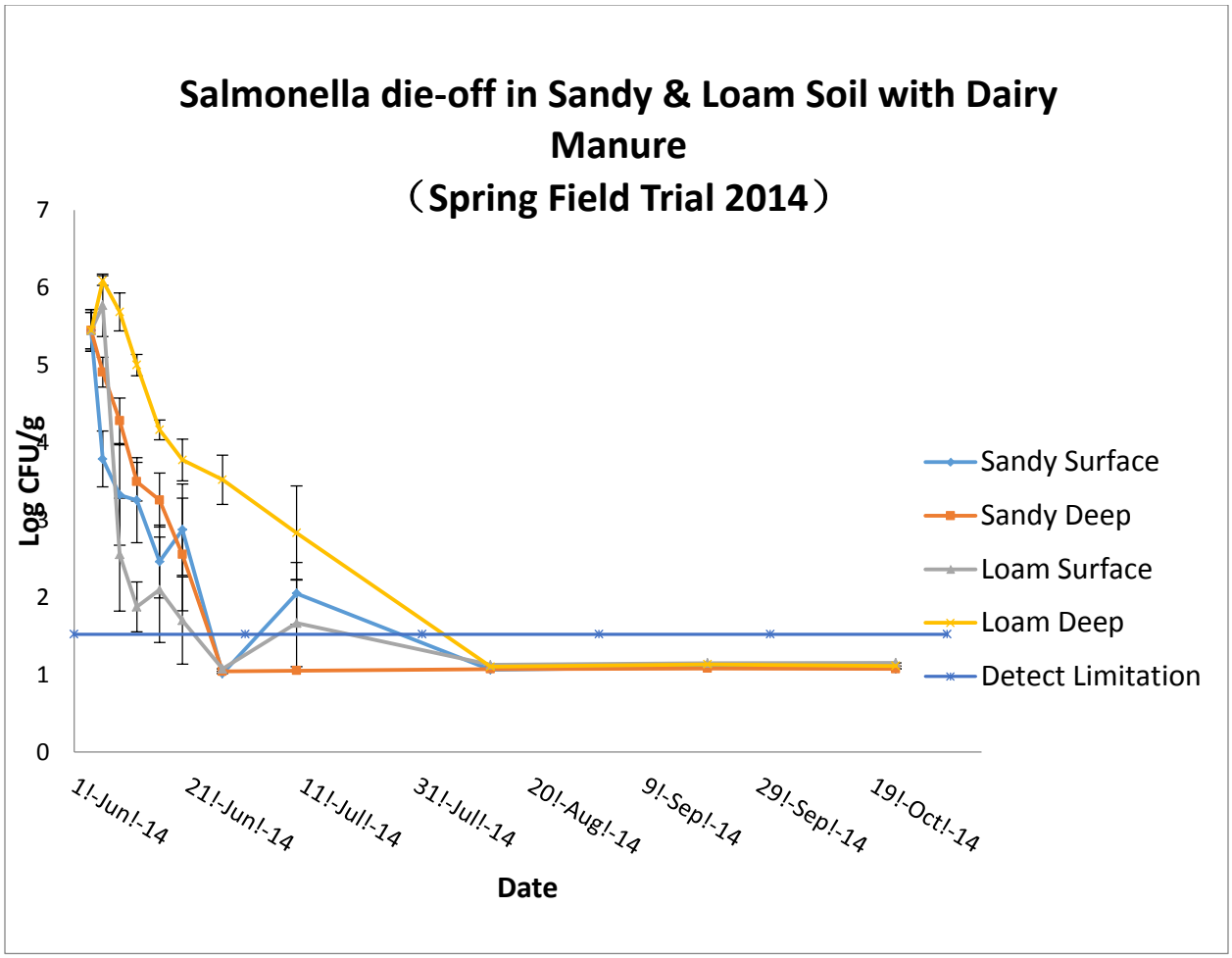


Figure 19: *Salmonella* levels in dairy manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

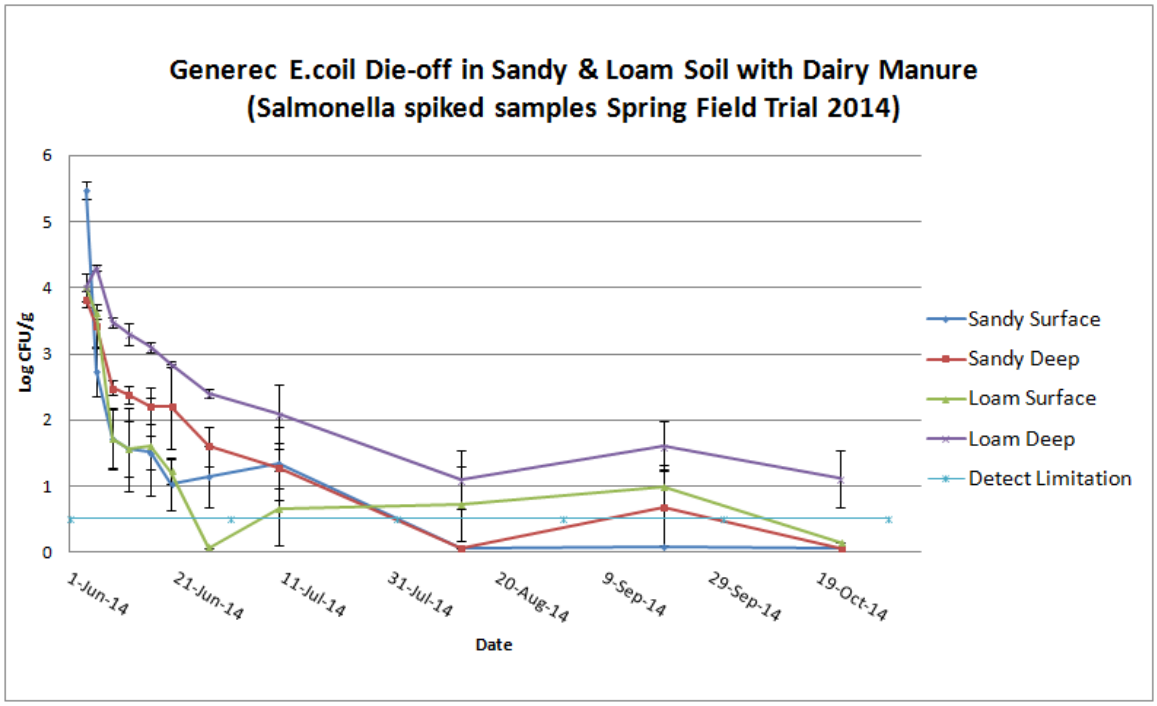


Figure 20: Generic *E. coli* levels in dairy manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

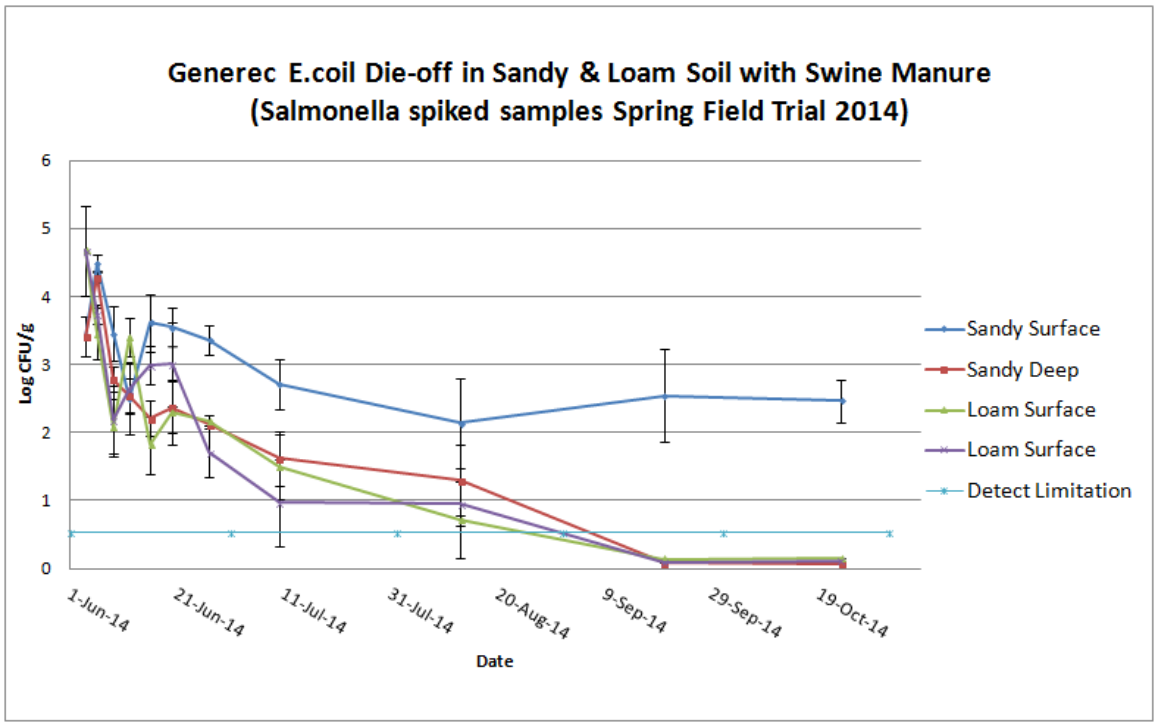


Figure 21: Generic *E. coli* levels in swine manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

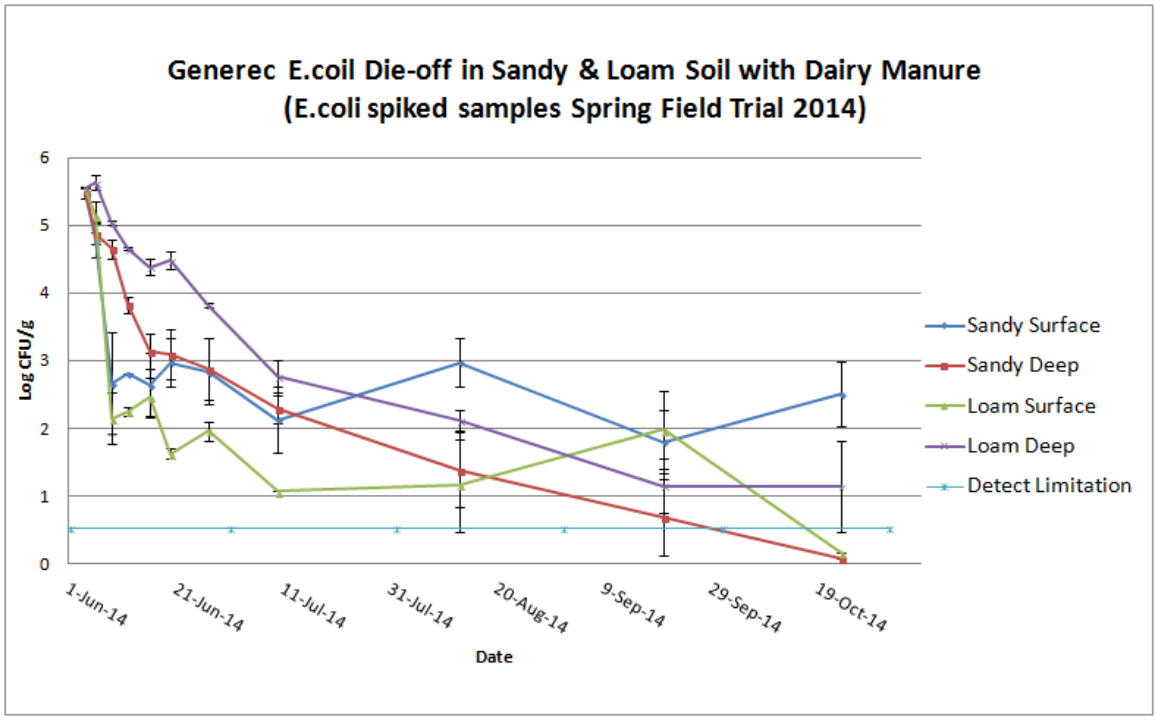


Figure 22: Generic *E. coli* levels in dairy manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

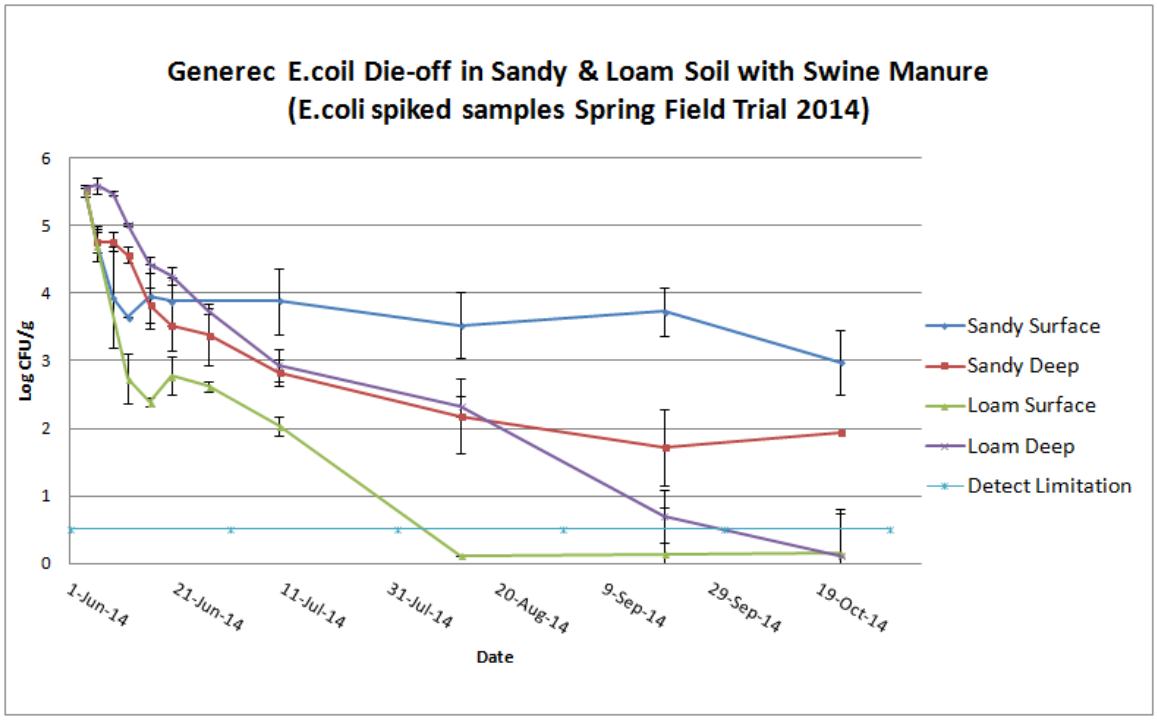


Figure 23: Generic *E. coli* levels in swine manure amended soil inoculated at depth (15 cm deep) or on the surface of loam or sandy loam soil.

Lab Trial Salmonella

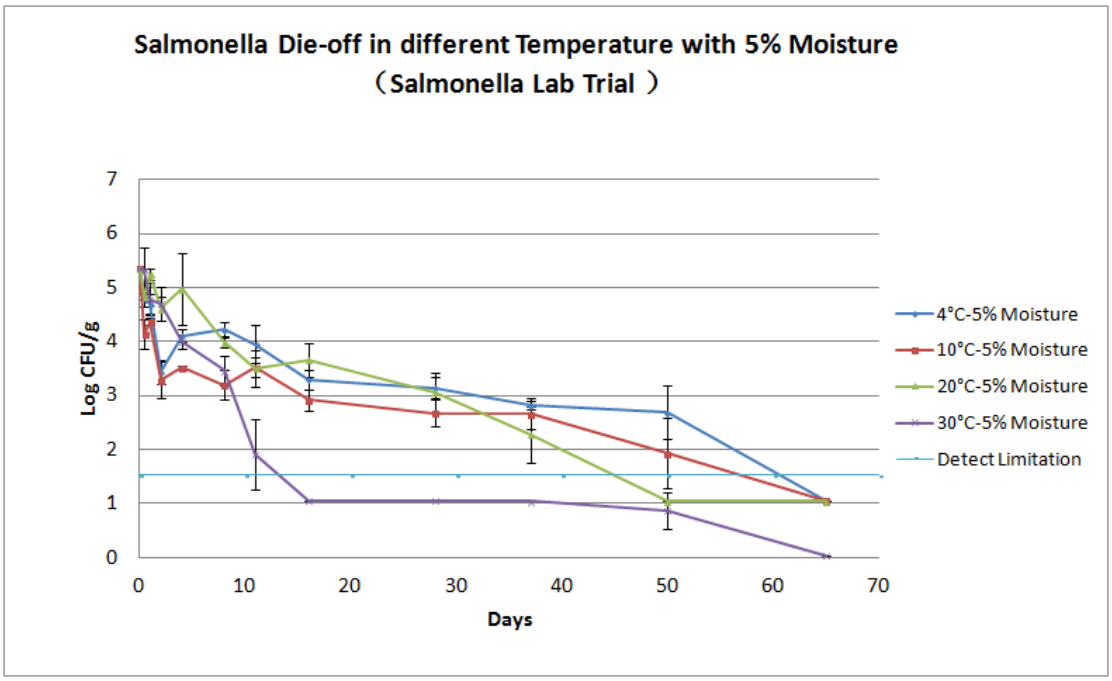


Figure 24: *Salmonella* levels in manure amended soil with microcosms held under different temperatures and relative humidity.

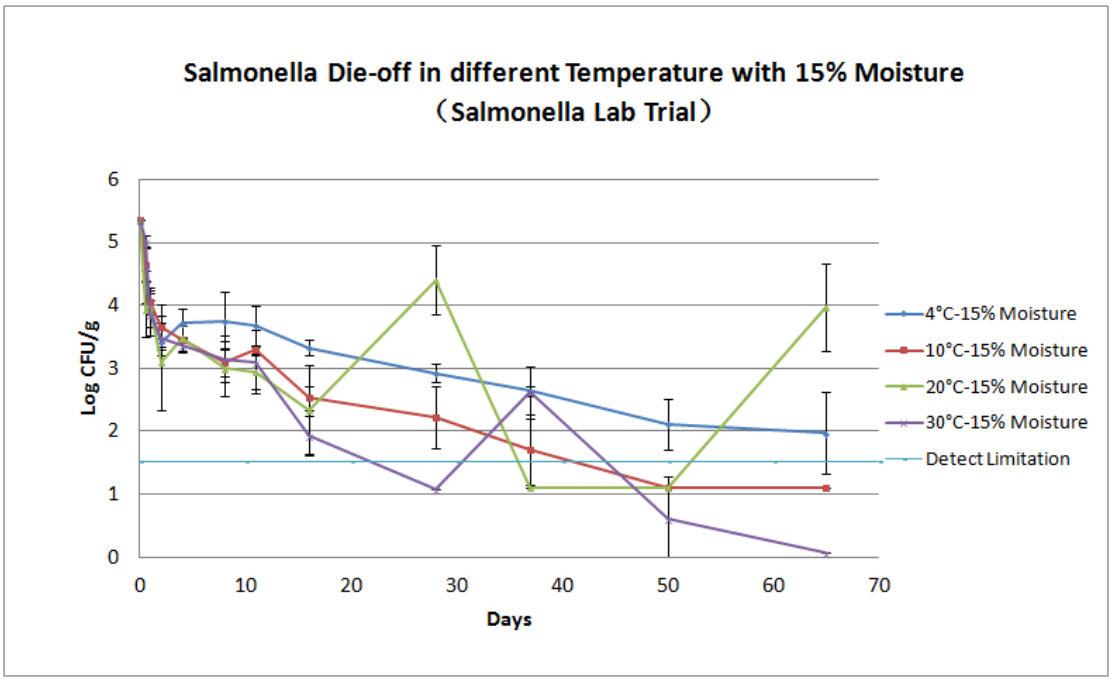


Figure 25: *Salmonella* levels in manure amended soil with microcosms held under different temperatures and relative humidity.

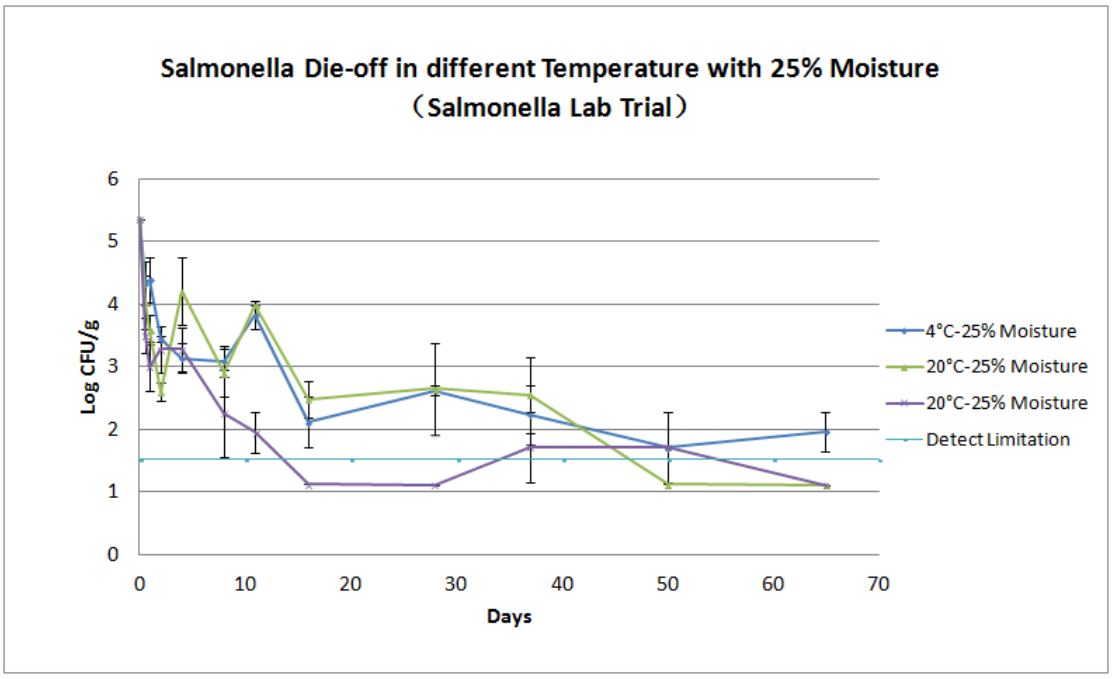


Figure 26: *Salmonella* levels in manure amended soil with microcosms held under different temperatures and relative humidity.

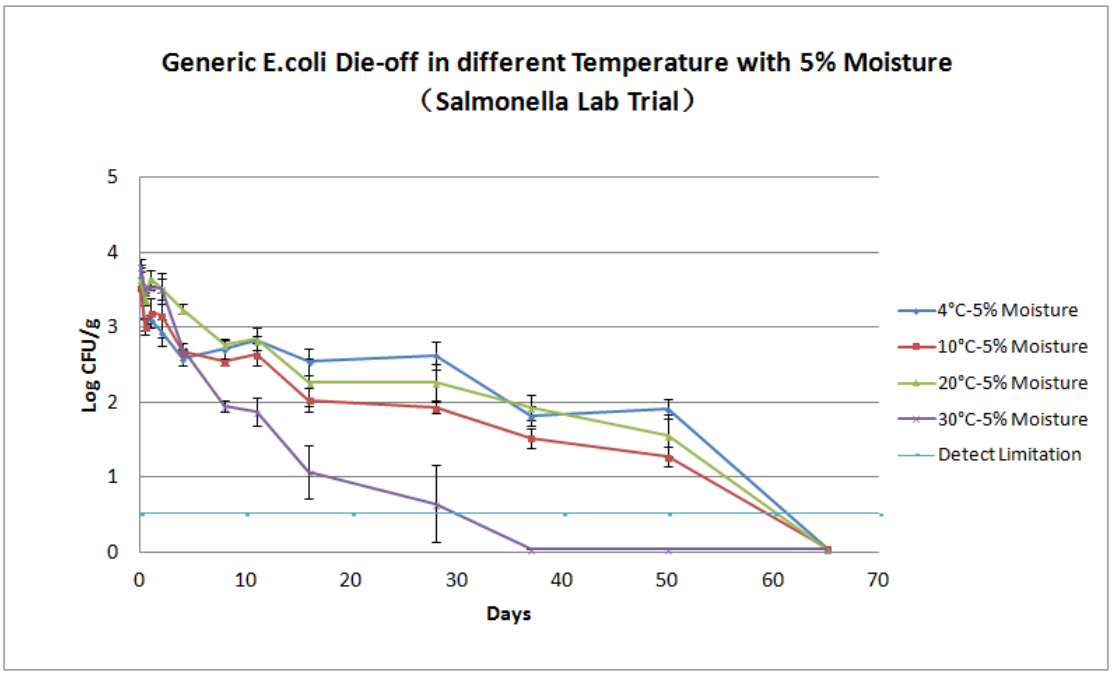


Figure 27: *E coli* levels in manure amended soil with microcosms held under different temperatures and relative humidity.

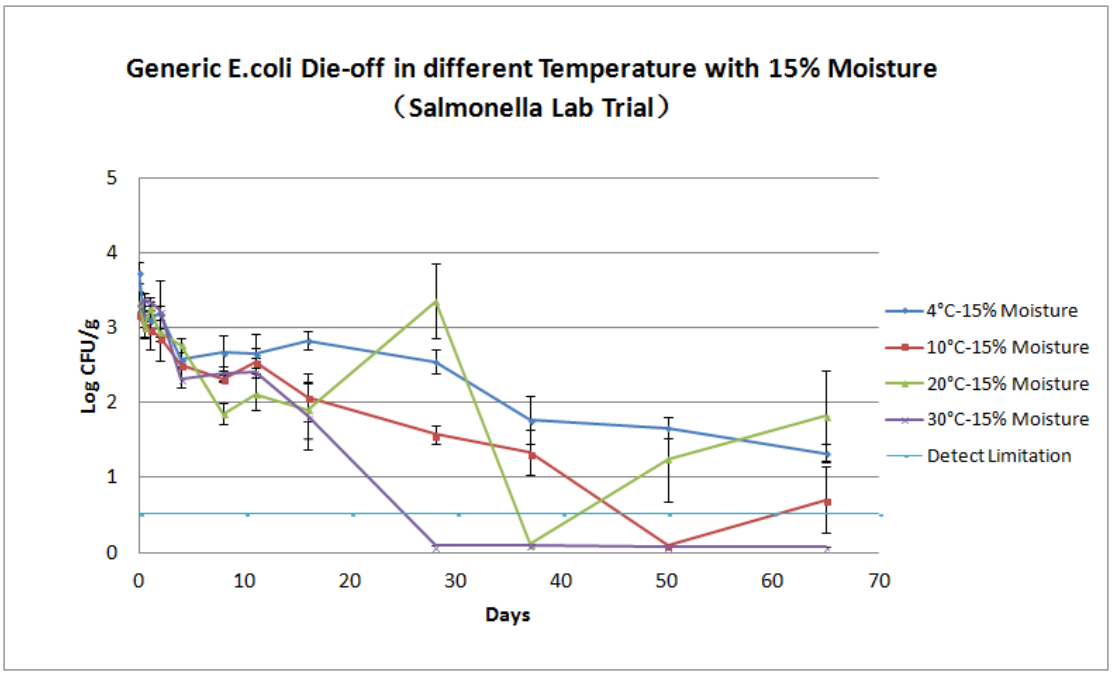


Figure 28: *E coli* levels in manure amended soil with microcosms held under different temperatures and relative humidity.

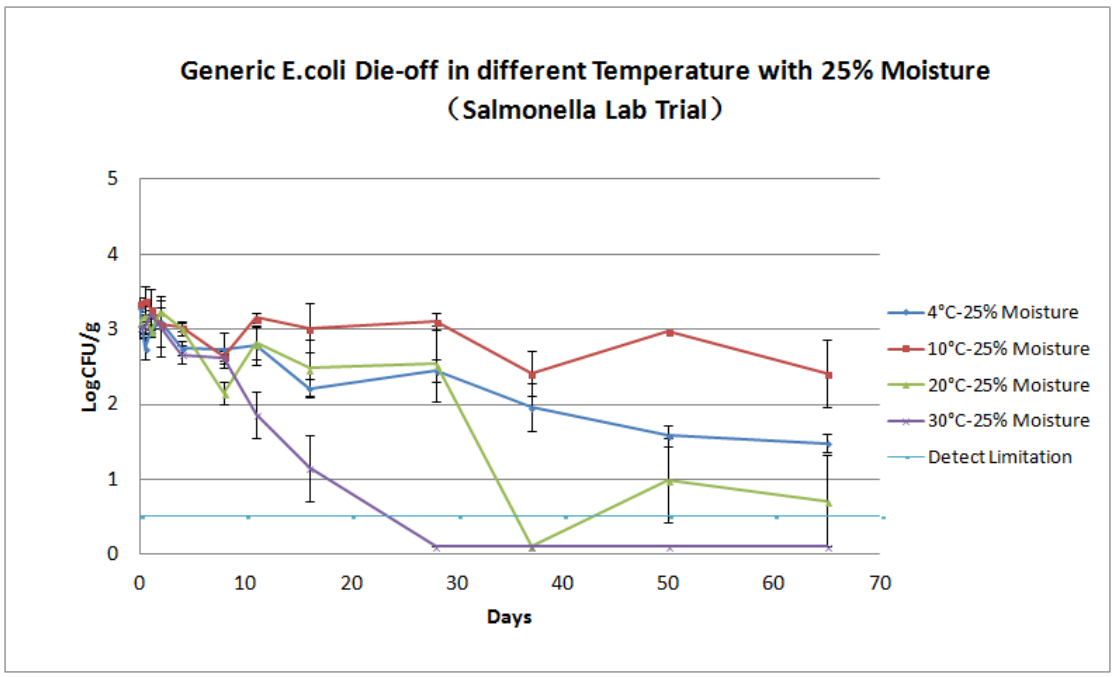


Figure 29: *E coli* levels in manure amended soil with microcosms held under different temperatures and relative humidity.

.

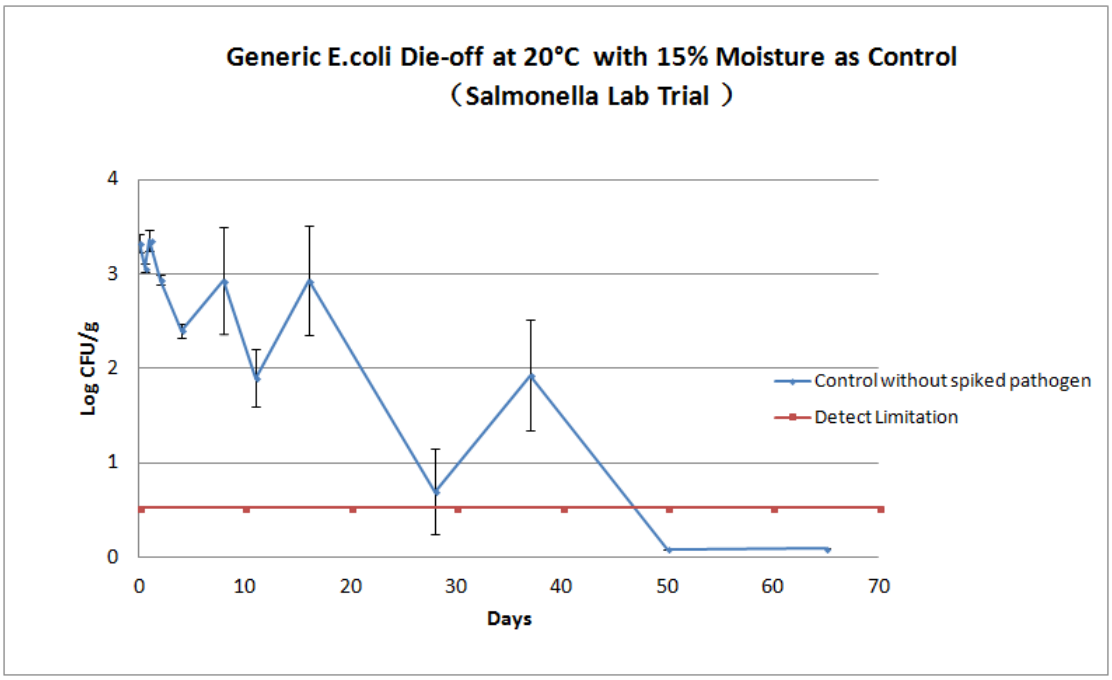


Figure 28: *E coli* levels in manure amended soil with microcosms held under different temperatures and relative humidity.

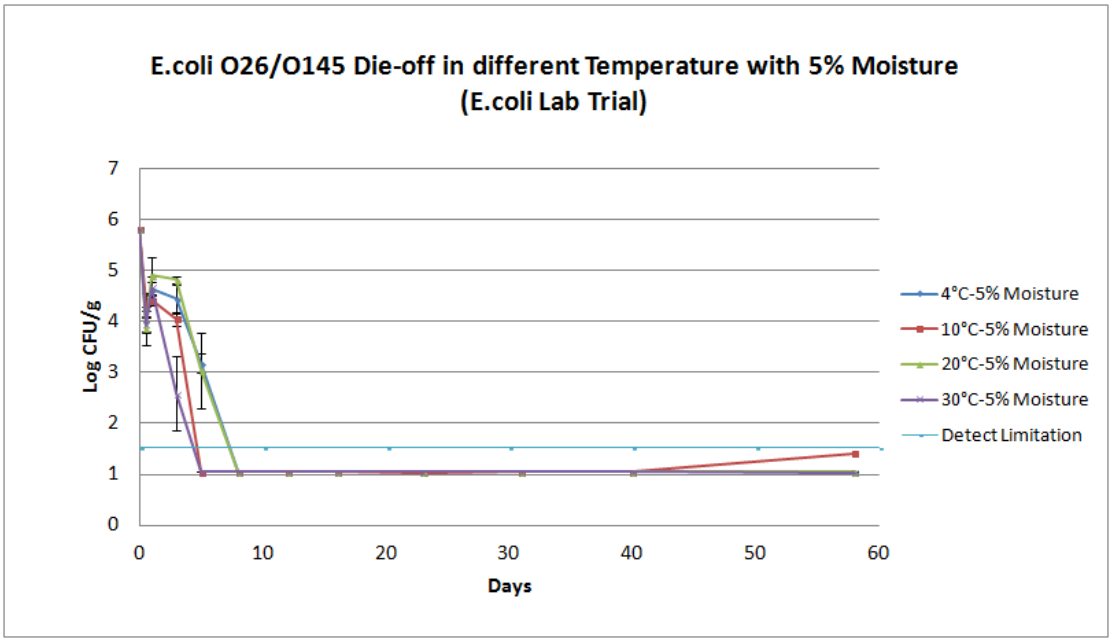


Figure 29: STEC levels in manure amended soil with microcosms held under different temperatures and relative humidity.

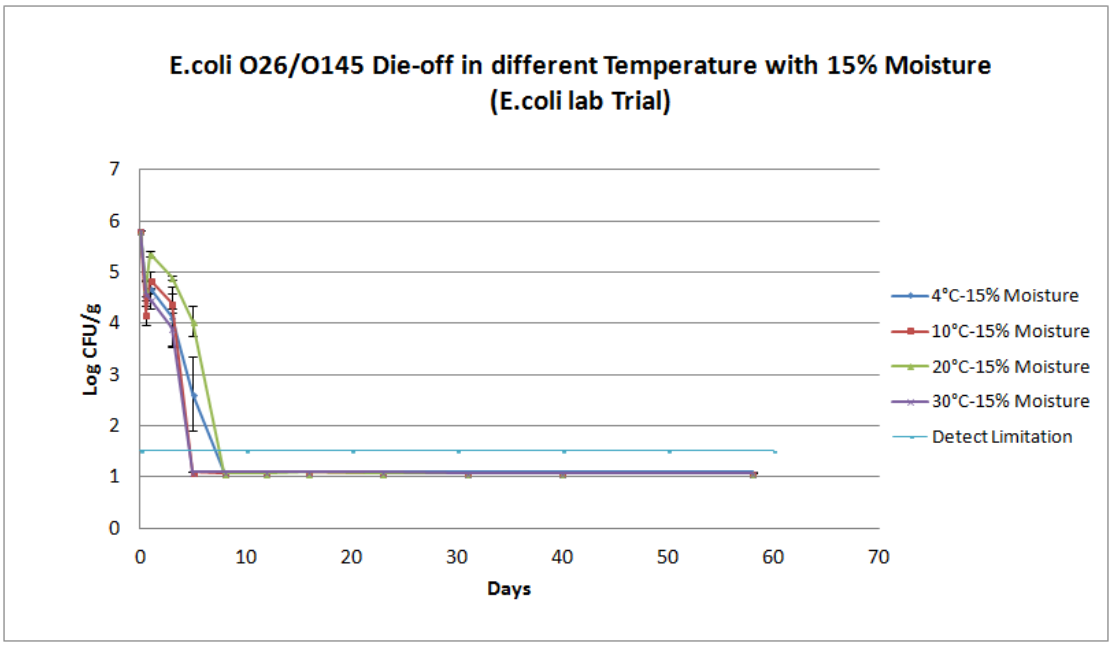


Figure 30: STEC levels in manure amended soil with microcosms held under different temperatures and relative humidity.

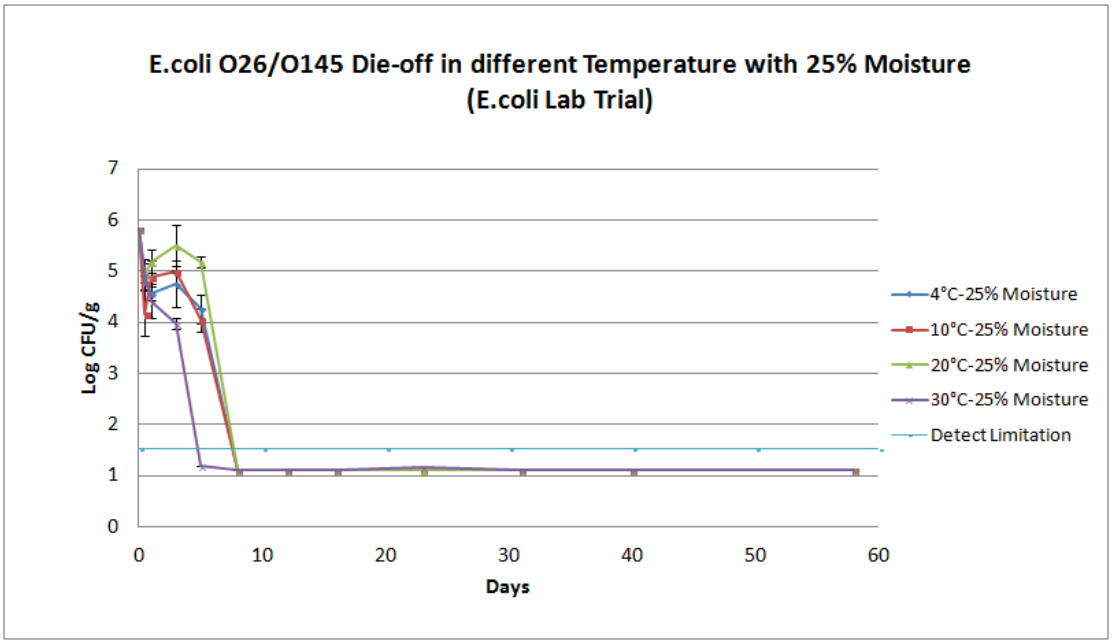


Figure 31: STEC levels in manure amended soil with microcosms held under different temperatures and relative humidity.

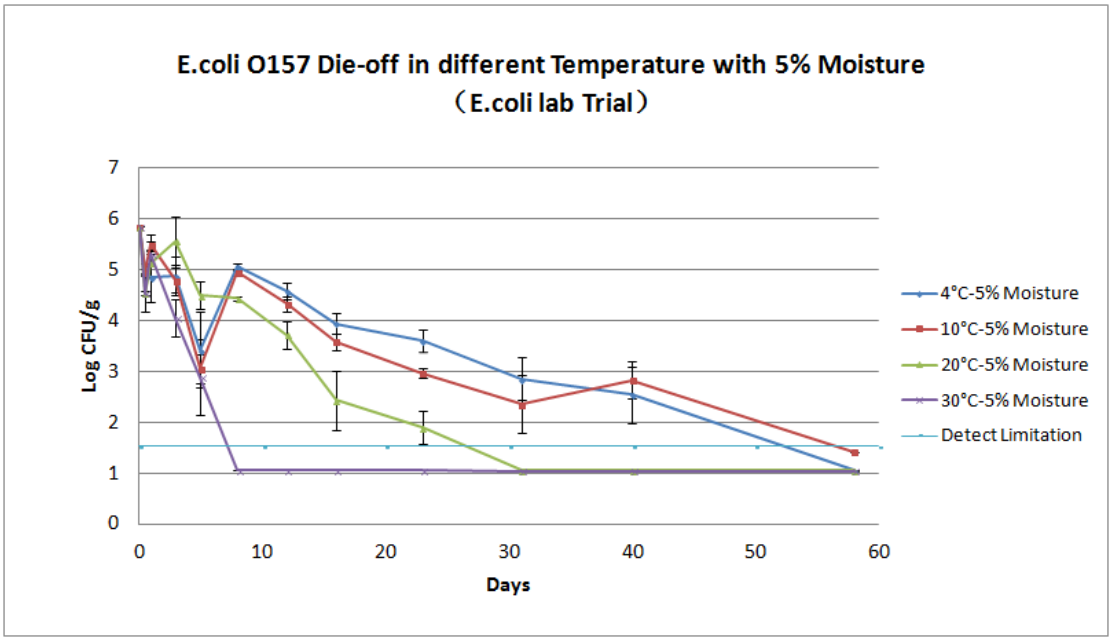


Figure 32: STEC levels in manure amended soil with microcosms held under different temperatures and relative humidity.

.

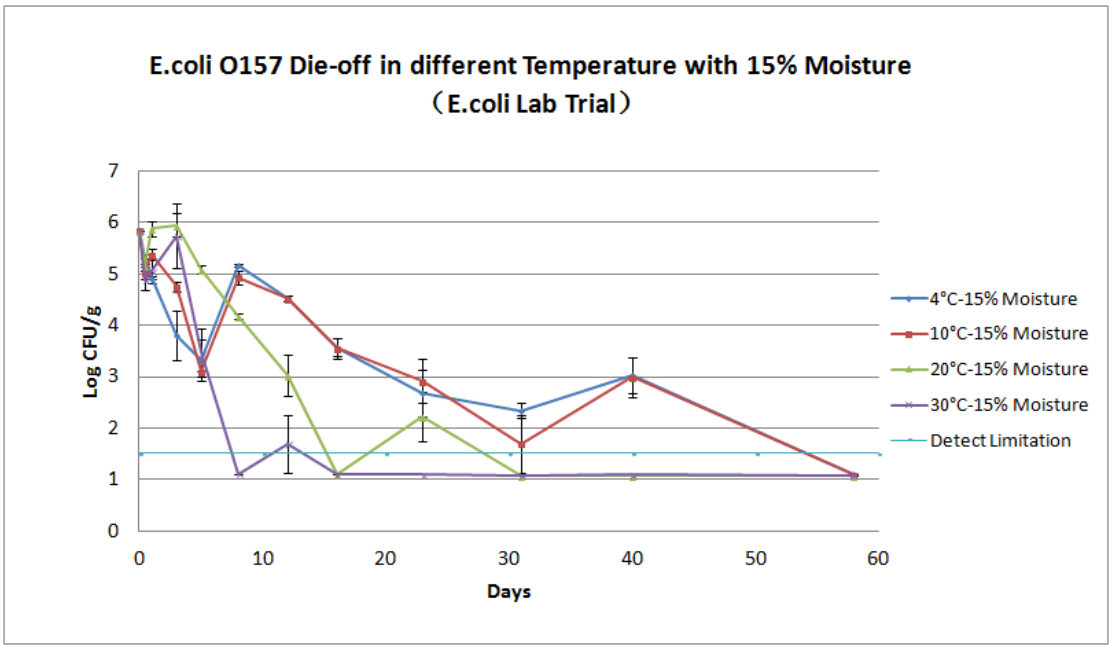


Figure 33: STEC levels in manure amended soil with microcosms held under different temperatures and relative humidity.

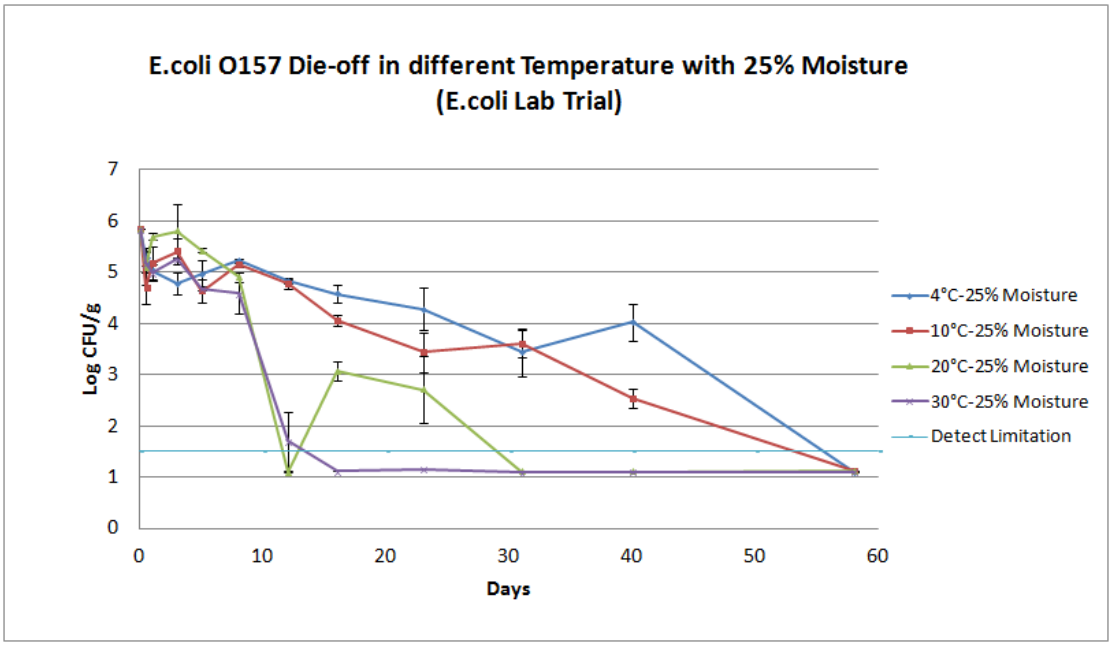


Figure 34: STEC levels in manure amended soil with microcosms held under different temperatures and relative humidity.

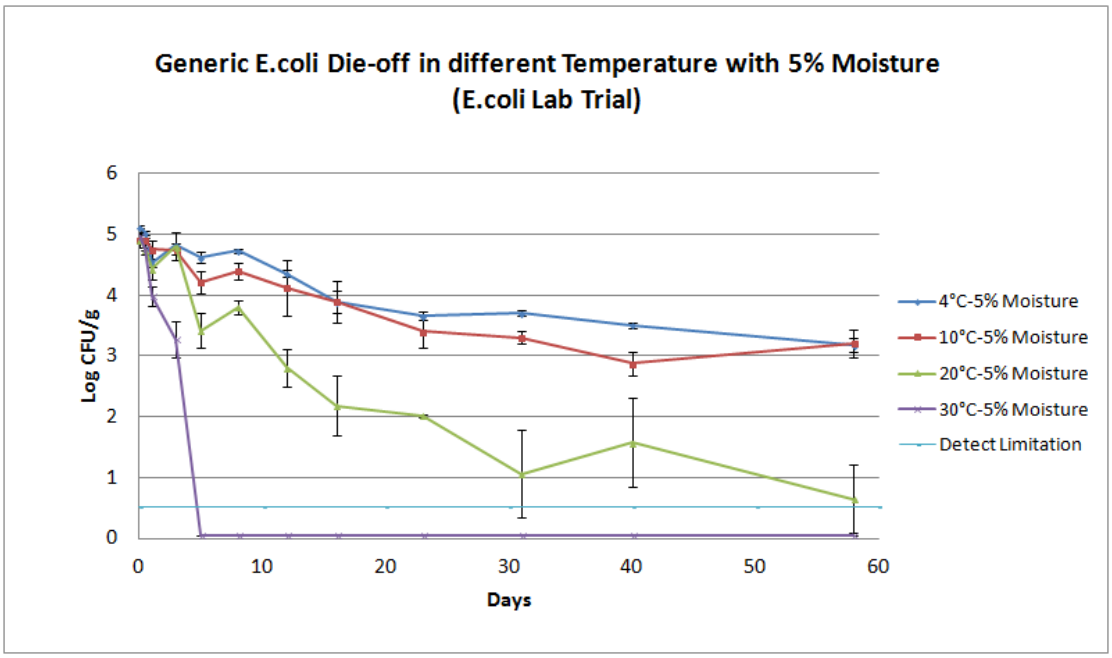


Figure 35: STEC levels in manure amended soil with microcosms held under different temperatures and relative humidity.

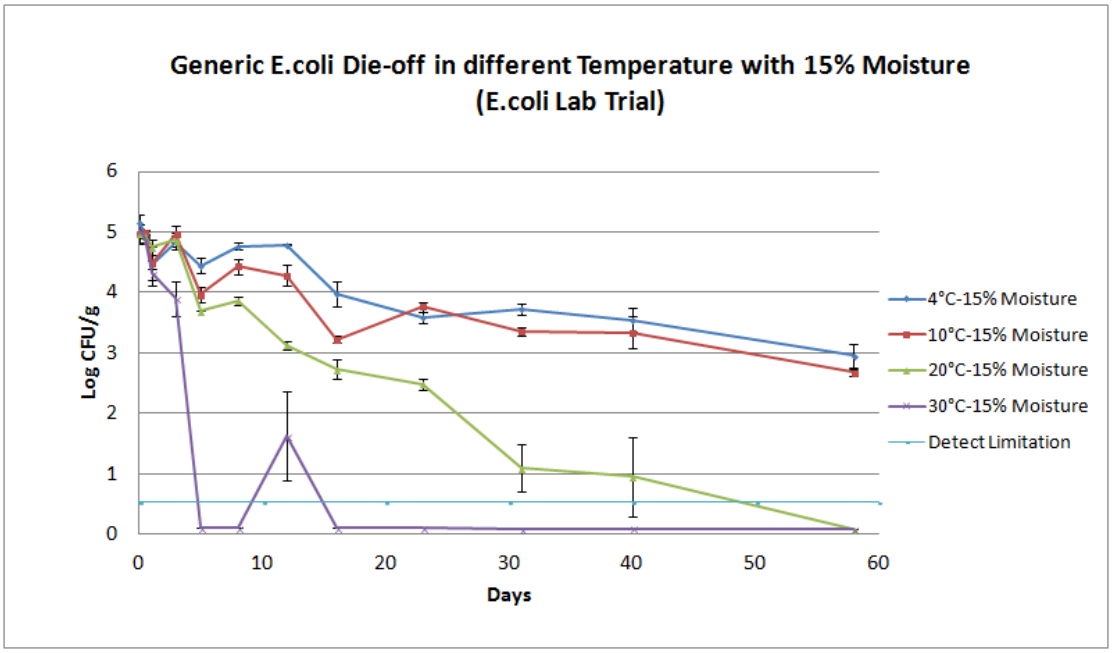


Figure 36: STEC levels in manure amended soil with microcosms held under different temperatures and relative humidity.

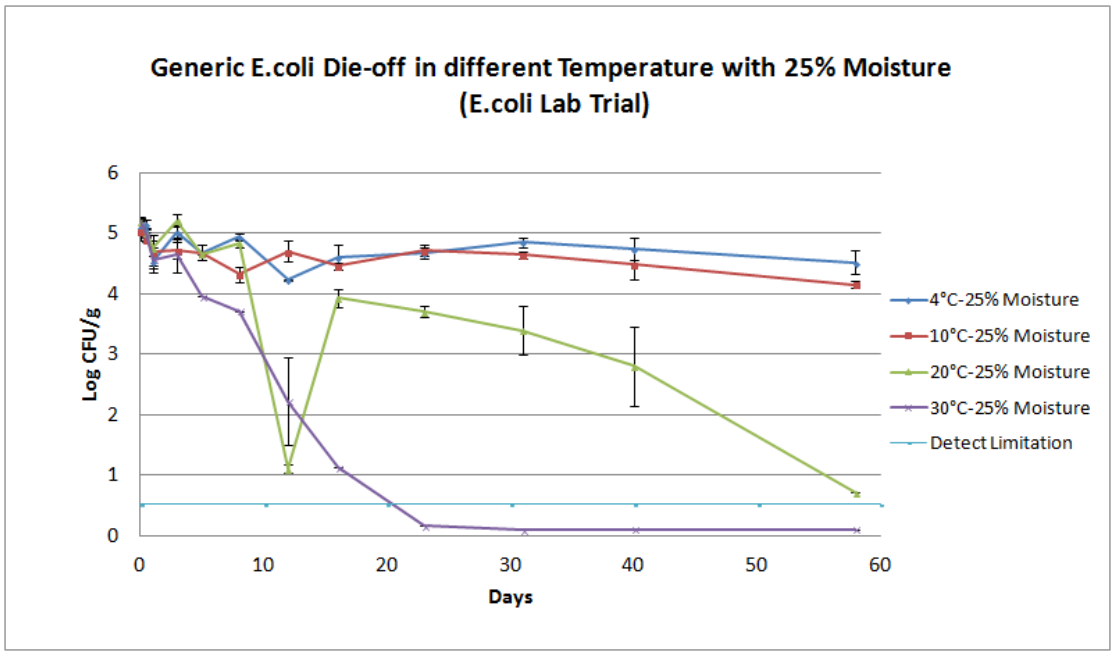


Figure 37: STEC levels in manure amended soil with microcosms held under different temperatures and relative humidity.

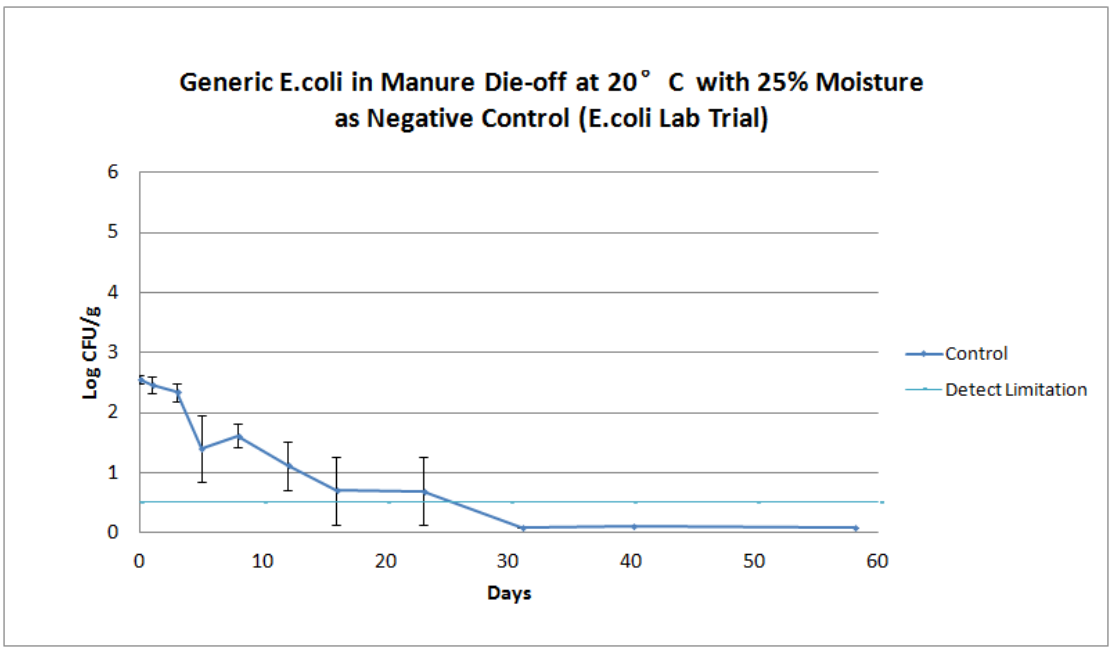


Figure 38: *E. coli* levels in manure amended soil with microcosms held under different temperatures and relative humidity.